A STUDY OF BACTERIAL L FORMS

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TO MY WIFE

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ABSTRACT

A survey of the early literature on aberrant bacteria has shown that what are now considered to be L forms or cell wall defective variants were well recognised entities long before Klieneberger drew attention to the presence of these microbial variants in the cultures of Streptobacillus moniliformis. The review of the more recent inter-disciplinary investigations carried out on L forms and other wall defective microbial variants has revealed that there is still a considerable lack of information on their biological and pathogenic properties.

Investigations carried out on ten strains of Salm. gallinarum have shown penicillin, glycine and a number of other substances used in the present work are capable of inducing L transformation in this bacterial species. Considerable variations occurred between the strains in their ability to respond to L transformation. Isolates from more recent clinical material were found to undergo L transformation more readily than the standard laboratory strains. Likewise it was easier to induce L transformation in the smooth than the rough strains of Salm. gallinarum. The ability of field isolates to undergo spontaneous L transformation without the aid of any known incitant is believed to be a new finding that has not been recorded in the Salmonella group of organisms.

A number of cultural and environmental factors

that aid in the transformation and propagation of these L forms were investigated and discussed. All ten strains of Salm. gallinarum produced only an unstable L growth on a solid hypertonic medium. A hitherto unrecorded finding is that in the two laboratory strains which, though unable to produce stable L growth, were able to do so in a liquid medium; furthermore the elimination of the serum requirement for stabilisation in a liquid medium is hoped will provide the basis for the future immunological and biochemical characterisation of the L forms of Salm. gallinarum.

Biochemical investigations on some of the L forms and revertants arising from these altered variants showed that they resembled in general their parental forms denoting absence of mutational changes.

Filtration studies have revealed that not all elements in L cultures are filterable.

Investigations on the ultrastructure of L forms of Salm. gallinarum show that the inducing agents used in the current studies bring about varying degrees of cell wall damage resulting in the production of cell wall defective variants not only varying in their sizes but also in the amount of cell wall layers retained.

The pathogenic properties exhibited by the unstable L forms of Salm. gallinarum were shown to be due to their reversion to the vegetative forms in vivo and the death of the experimental host was ascribed to the endotoxic properties of the organism.

PART I

HISTORICAL BACKGROUND AND REVIEW OF THE LITERATURE

PART I

HISTORICAL BACKGROUND

The ability of bacteria to perpetuate themselves by a myriad of structures was clearly demonstrated in the discovery of L forms by Klieneberger in 1935, although at that time she erroneously regarded these atypical growths as pleuropneumonia-like organisms. The concept that bacteria could exist, transform and reproduce in an altered state dates back to more than sixty years before Klieneberger's discovery of Ll organisms.

One of the earliest descriptions of the transformation of bacteria is perhaps that of Burdon-Sanderson who in 1871 mentioned the transformation of spheroids into rod forms and also the possibility of bacteria developing from ultramicroscopic bodies. Billroth (1874) stated that, besides spores, minute coccoid bodies played an important part in the multiplication of bacteria.

Globular structures, coccoid forms and rounded bodies have been encountered in Mycobacteriaceae by Neisser (1881), Hansen (1882), Malassez and Vignal (1883), Babes (1883), Lutz (1886), Bordoni-Uffreduzzi (1888), Karawachi (1925) and Kahn (1929). The occurrence of coccoid and ovoid structures amongst anthrax bacilli has been recorded by Klien (1883),

De Barry (1884), Ehlers (1884) and Crookshank (1896). The presence of spindle-shaped and irregularly swollen bodies and coccoid forms in Streptobacillus moniliformis cultures had been reported by Tunnicliff (1916), Levaditi, Selbie and Schoen (1932) and Strangeways (1933). Hauser (1885) observed large globular and club-shaped forms measuring 3-7 μm in his Proteus cultures. These were described by him as involution forms but he also observed that they could be stabilised and were able to multiply in successive transfers.

Ferran (1885) made some interesting observations on the development of Vibrio cholerae in hanging drop preparations. He observed not only large round bodies which he called 'oogones', but also smaller rounded bodies called 'pollinodes' which conjugated with the 'oogones' to produce oospores, which in turn produced granules. These granules grew into 'corps muriformes' which ultimately gave rise to spirilla that divided to form classical Vibrios. Ermengen (1885) concurred with Ferran's findings in as far as granules and 'corps muriformes' were concerned. Dowdeswell (1890) described the phases of development of Vibrio cholerae in wet preparations. His findings suggested a much less complicated evolutionary life cycle than did those of Ferran, although there were many features in common.

Firtsch (1888), in his studies on Vibrio proteus, described large plasma globules containing granules. He observed that these large structures, spherical in shape, arose from the fusion of filaments. According to Klieneberger-Nobel (1951), Firtsch was probably one of the earliest bacteriologists to observe the production of L elements. Zarniko (1889) observed club-shaped cells and ovoid forms in the diphtheria bacillus. Zettnow (1891) presented photographic evidence of budding and regenerative elements in bacteria referring to them as globules that separated from the parent bacteria. Lafar (1887) had suggested that organisms which did not conform to normal morphology be classified as involution forms if their physiological activities relating to growth, multiplication and motility were reduced or absent. Russell(1892) found large globular bodies in ageing cultures of B.granulosus. He did not consider that these were involution forms since they were capable of multiplying and forming spores. Similar observations concerning the ability of spherical bodies and giant forms to multiply and transform have been recorded by Schürmayer (1898) for avian type tubercle bacilli, Galli-Valero (1903) for B.pestis, Hammerl (1960a & b) for Vibrio cholerae and Almquist (1893, 1907) for B.typhosus and B.coli. Rothert (1902), in his dissertation on "Degeneration

and Regeneration of Bacteria", stated that during their degenerative stages, bacteria produced resistant granules which were capable of regeneration. These granules were observed by him in the following species of bacteria:-

Staphylococcus pyogenes aureus, B.coli, Streptococcus pyogenes, B.typhosus, B.pneumoniae, B.capsulatus (Pfeiffer), B.rubidis, B.arborescens, Vibrio cholerae and Vibrio milleri.

The presence of altered structural variants in vivo was reported as early as in 1878 by Letzerich who saw large spheroids in the blood taken from typhoid patients. These, which he called 'plasmakulgen', later gave rise to rods within themselves. Klien (1894) reported the occurrence in diphtheric membranes of spherical and cuboidal types of diphtheria bacilli as well as altered bacillary forms with terminal knobs or clubbed ends. Pfeiffer in 1895 noted the in vivo transformation of bacteria into spheroids when he examined the peritoneal fluid obtained from guinea pigs which had been injected intraperitoneally with vibrio cultures.

That the constancy in shape of an organism could be altered was first shown by Buchner (1882) with B.anthracis. He succeeded in changing the morphology of the rod-like bacilli into isodiametric cells by adding large amounts of sugar to the growth medium. Klien

(1894) stated that the shape in which a bacterial species presents itself depends both on the medium in which it grows as well as the inherent characteristics of the organism. Albrecht and Ghon (1920) pointed out that the addition of 5 per cent. glycerol or sugar to the medium was useful for the study of abnormal forms of B.pestis. The effect of the temperature at which organisms were cultivated on the induction of morphological variants has been described by Klien (1883 & 1885 a & b). He noted that the Bacillus anthracis, when grown on gelatine media at room temperature (15° - 20°C), became spherical or ellipsoidal with the cells arranged in rows to form chains. These cells had granules and were capable of multiplication, with prolonged incubation many of these were converted into threads of anthrax bacilli. He distinguished these forms from spores of anthrax. In his studies on the aetiology of asiatic cholera, Klien (1885 a & b) observed that Vibrio cholerae when grown on agar at room temperature swelled up owing to the appearance of vacuoles within the cells. As these vacuoles increased in size the bacterium ultimately assumed a circular shape and then, by division along its diameter, produced two comma-shaped bacilli. When the typical comma-shaped forms were grown at high temperatures of 30°- 40°C they multiplied only by transverse division but, when they were kept at room temperature, gradually transition to the circular

forms took place. Klien's illustration of these circular forms is not altogether convincing as some of his so-called 'corpuscles' are undoubtedly the result of two comma-shaped bacilli being attached to each other at both ends to form a ball-like structure with a large central vacuole. It was shown by Almquist (1893) that E.coli and B.typhosus when grown at low temperatures (10°- 11°C), produced very small round bodies by fragmentation as well as by budding and these, when transferred to fresh broth medium, produced normal rods. In a subsequent paper (Almquist,1909) he reported that the agents of cholera, typhoid and dysentery grown on agar slopes at 10°C first produced large forms which gave rise to budding, globular structures. If the large forms were transferred within a certain time to a new medium at higher temperatures, globular structures were produced extremely quickly. The globular forms of Vibrio rapidly produced bacilli or could multiply as globular forms, but in the case of typhoid and paratyphoid bacilli, the globular bodies produced finer 'spore-like structures' which then transformed into rod forms. In the latter group of organisms the globular forms were also able to perpetuate themselves as globular bodies. Durham (1898) and Maassens (1904) have also reported the effect of temperature on the production of abnormal growth

variants in bacteria.

The influence on the cell morphology of the hydrogen ion concentration in the growth substrate was studied by Wasserzug (1888 a & b) who noticed that B.prodigiosus always produced rods and thread-like forms in an acid broth but coccoid and spirillar forms in alkaline broth. Haffkine (cited by Hankin and Leumann, 1897) recommended the use of agar media having a well marked alkaline reaction for the production of involution forms of B.pestis. The morphological response of diphtheria bacilli to ionised acidity of the culture medium was described by Bunker (1917). Very distinct morphological forms of the organisms developed in media of different reactions. He reported that very acidic media gave large irregular forms in pure culture. Very alkaline media yielded pure cultures of minute triangular forms. The transformation of B.typhosus, B.coli and Shigella spp. to pleomorphic forms has also been observed in acidic broth medium by Hort (1917).

The use of high salt concentrations in the culture medium to secure a quicker development of pleomorphism has been investigated by a number of early workers in this field. Hankin and Leumann (1897) inoculated B.pestis on media containing 2.5 to 3.0 per cent. sodium chloride to obtain involution forms at an

incubation temperature of 37°C. With a lowered temperature of incubation (30°C) these forms appeared more slowly after two or more days. These authors also demonstrated that by increasing the salt concentration morphological distortions were greatly increased. They observed large spheres and pear-shaped bodies. With lowered salt concentration the bacilli merely swelled up and no gross alterations in cell morphology were observed. Matzuschita (1900) produced photographic illustrations of B.coli and B.typhosus grown on salt agar under similar conditions. In both the species grossly enlarged filamentous forms with bulbous swellings along their length were present. It appears from these photographs that in B.coli the changes were more marked compared with the induced changes in B.typhosus strains. This author also published photographs of spherical forms of B.anthracis grown on salt agar. Analogous results with various other species of bacteria have been recorded by Rosenfeld (1901 a & b),Stefansky (1902), Almquist (1904), Maassen (1904) and Davis (1907).

Hata (1908) used various amounts of calcium chloride, magnesium chloride and sodium chloride to produce morphological variants with B.pestis, Salm.typhi, B.dysenteriae, B.coli and Vibrio cholerae, Casagrandi (1901) obtained branched and filamentous forms by growing B.typhosus in media containing gypsum.

Lithium salts were also very effective in producing globular and branched forms (Gamaleia, 1900; Maassen, 1904).

Hadley et al. (1931) found that adding lithium chloride to growth media had the effect of producing dissociant colonies of Shigella bacilli. The 'G' colonies they thus obtained consisted of a variety of morphological types ranging from granules and coccal forms to large globules and grossly deformed rods. Maassen (1904) used salts of rubidium, calcium, magnesium, strontium and barium to produce involution forms. Walker and Murray (1904) observed morphological changes in bacteria grown in media to which small percentages of various dyes were added. These morphological transformations occurred with constancy and consisted of a complete conversion of normal rods into threads or filaments which showed no segmentation; the filaments were sometimes branched, these effects were produced in B.coli, B.typhi and Vibrio cholerae by methyl violet, methyl green and methylene blue, but the best results were obtained with a 0.2 per cent. saturated solution of methyl violet. Sub-cultures of these forms into media containing no dyes produced normal rods. These authors' description of the tiny colonies on methyl violet agar with a 'central nucleus' suggests that they were probably L type colonies. Their microphotographs not only show

swollen filaments and thread-like structures but many transitional forms - bulbous forms, spindle-shaped elements, large globular and ring-shaped structures. Revis (1912) used malachite green to transform B.coli into abnormal morphological types.

Péju and Rajat (1906 a & b) added urea to the growth substrate to produce filaments, pear-shaped bodies and spindle-shaped forms from Salm.typhosus. Wilson (1906) made similar observations with B.coli, B.typhosus, B.enteritidis, Bacillus pyocyaneus and Klebsiella pneumoniae; with B.pestis the growth on urea agar produced coccoid and rounded forms but no filaments were produced. Some of the rounded forms were attached by means of thread-like structures to similar round bodies or bacillary forms. The occurrence of large circular swollen bodies with weakly staining periphery and a deeply staining centre was also described. No changes were observed with several strains of Staphylococcus spp. and streptococci. Péju and Rajat (1906c) and Rajat and Péju (1906) transformed B.typhosus into various structurally altered shapes by the use of potassium iodide. Many other compounds such as copper sulphate (Garbowski, 1907 a & b), potassium dichromate (Thiercelin, 1903), carbolic acid (Cacae, 1903) and naphthol and creosote (Charrin, 1889) have been used to produce similar effects.

The findings of Schultz (1901) suggested that the accumulation of metabolites in the growth medium induced granule formation. In her studies on the plague bacillus, she found that in old cultures large numbers of granules were present which, when transferred to a fresh medium, at first produced large numbers of globular bodies which subsequently produced rods.

The effects of ultra-violet radiation on bacterial cell morphology has been reported by Henri (1914). By exposing a watery suspension of B.anthracis to ultra-violet rays, she noticed that the cells were either killed or remained normal but that some of these gave rise to colonies which consisted of bacteria which were ellipsoidal, ovoid or even spherical in shape. Many of these spherical bodies were able to revert to the rod forms. Some of these spherical forms did not revert but stabilised as spherical bodies. Her descriptions and photographic illustrations of these forms leave very little room to doubt that what she had observed were in all probability the L forms of B.anthracis.

Induced changes in the morphology of bacteria as a result of interaction between various species appeared to have been investigated fairly early in the study of bacterial polymorphism. Lorenz (1892) reported that when B.erysipelatous suum was inoculated into a filtered broth in which B.suisepticus was grown;

an actinomyces-type of growth was obtained which on gelatine medium reverted to rod forms similar to those found in the animal body. Metchnikoff (1894) observed that in Vibrio cholerae cultures which contained a coccus, club-shaped and other abnormal forms developed. Kurth (1898) observed that the diphtheria bacillus was most susceptible to morphological changes when in the presence of other organisms. When this organism was grown in the presence of a streptococcus, the diphtheria bacillus assumed very short forms which could hardly be distinguished from cocci. Smirnow (1908) made similar observations with this organism. He found that when the diphtheria bacillus was grown in the presence of a coccus (presumably meningococcus) or Streptococcus pyogenes, coccoid and involution forms of B.diphtheriae were produced which subsequently transformed into typical beaded forms. Wolff (1908) found club-shaped and spindle-shaped transformation of Streptococcus lactis (Bact.guentheri) grown in symbiosis with B.putrificus. The remarkable consistency of these results does not rule out the possibility of contamination of the cultures, but nevertheless it provides sufficient evidence not to exclude totally the possibility of morphological transformation as a result of interaction between different species of bacteria. Dienes' recent observations (Dienes,

1970 a & b) that Bacillus Y greatly enhances the reversion of L forms of Haemophilus influenzae, Proteus spp. and streptococci into bacillary forms lends credibility to many of these early observations. Following the early morphological observations on altered and induced variants, attention was focussed on filterable forms of bacteria in relation to disease processes as well as their part in the life cycle of bacteria.

Much (1907 a & b, 1908) demonstrated that the causative agent of tuberculosis could exist in two forms, (a) the classical acid-fast bacilli which were difficult to demonstrate in tuberculous lesions and (b) non-acid-fast, granular forms and tiny slender rods - the only forms found in lesions. Fontes (1910) suggested that Much's granules were filterable. He observed that Berkefeld filtrates of tuberculous material injected into guinea pigs gave rise to enlargement of the lymphatic glands and spleen and that in the lymphocytes of the affected glands 'Gram fast' granules of Much could be demonstrated but not acid-fast bacilli. He came to the conclusion that the granules of Much constituted a stage in the development of the tubercle bacillus. Vaudremer (1921) grew tubercle bacilli in potato broth medium and was able to get non-acid-fast filaments which contained minute granules. He maintained that these granules were

filterable and, on suitable media, gave rise to colonies of typical acid-fast bacilli. In a subsequent paper (Vaudremer, 1923) he reaffirmed the existence of filterable forms of tubercle bacilli. Hauduroy and Vaudremer (1923) were also able to get filamentous granular growth from filtrates of Mycobacterium tuberculosis grown on potato medium. These filtrates contained no acid-fast bacilli. Valtis (1924) confirmed the observations of Fontes by repeating the latter's experiments. The Berkefeld and Chamberland filtrates did not produce cultures but, when injected into guinea pigs, caused enlargement of the lymphatic glands and in some of them acid-fast bacilli could be demonstrated. Durand and Vaudremer (1924) appear to have obtained analogous results. Calmette, Valtis and Lacomme (1926) have described that 'ultra microscopic' forms of tubercle bacilli were able to pass through placental barriers and infected the developing foetus. Morin and Vatis (1926) reported the existence of filterable forms of Mycobacterium johnei. Kahn (1929 & 1930) investigated the developmental stages of the tubercle bacillus in micro-droplets containing between 1 to 6 organisms. According to him the human tubercle bacillus did not divide solely by binary fission but by a series of complicated reproductive processes. He observed that the rod forms of the bacilli, after a few hours' incubation

at 37°C, showed zones of darker areas; twenty-four to seventy-two hours later these rods segmented into 3 to 4 ovoid bodies. Sometimes globoid forms were produced. These forms did not develop into rods but sub-divided to form diplococcal elements which were further reduced to a mass of dust-like particles and granules. These remained as such for varying periods of time but ultimately gave rise to extremely tiny rods which further elongated and thickened to mature acid-fast bacilli. Kahn postulated that these evolutionary stages were probably induced as a result of artificial cultivation and concluded that such a sequence of events was unlikely to take place in vivo. He was unable to demonstrate the filterability of these granular forms, postulated by earlier investigators. Mellon and Fisher (1932) used an acid-fast tubercle bacillus to produce filterable granules. They claimed that these granules, when transferred to appropriate media, transformed to non-acid-fast diphtheroid forms which differed from the parent organism in cultural characteristics. In a subsequent paper Mellon, Richardson and Fisher (1933) described a seven stage life cycle for the avian tubercle bacillus in which the non-acid-fast filterable granules formed a part of this life cycle. The granules, according to them, gave rise to coccal forms which in turn produced diphtheroid forms; these non-acid-fast diphtheroid

forms produced 'spore-like gonidia' which subsequently transformed into R forms and finally S forms of the bacilli. Further evidence of the existence of these granules was provided by Kahn and Nonidez (1933) but they were once again unable to confirm the filterability of these granules. They concluded that the granules were not involution forms as suggested by Bergel (1914) and Ørskov (1932) but active growth components of Mycobacterium tuberculosis colonies. The findings of Kahn and Nonidez (1933) were partially supported by Hauduroy's observation (1946) that the pellicles in liquid cultures of Mycobacterium tuberculosis contained both acid-fast and non-acid-fast forms and the latter represented the younger stage of the two.

Histological evidence of the existence of these granules was provided by Pagel (1934) who found granules in old caseous and calcified tuberculous lesions. Alexander-Jackson (1945, 1947) described a zoogleal form in Mycobacterium tuberculosis which she demonstrated by means of a special staining technique. Using this staining method she was able to demonstrate not only acid-fast forms but also semi-acid-fast and non-acid-fast forms (rods, granules and zooglea). These zoogleal forms were sometimes the only forms found in more advanced cases of leprosy and tuberculosis. She postulated that the zoogleal forms

may be a phase in the development of Mycobacterium tuberculosis and Mycobacterium leprae.

Many other investigators were unable to confirm the existence of either the filterable forms of the tubercle bacillus or the granules of Much. Bittrof and Momose, cited by Griffith (1930), were unable to demonstrate the non-acid-fast forms or granules of Much in tuberculous lesions. Bergel (1914) considered that the granules of Much arose from the action on the tubercle bacilli of lipoidal substances in the blood and lymph and as such the granules were degenerate elements. Ørskov (1932) also regarded the small forms and granules of Much as products of degeneration and crystallization found in old cultures. Cooper and Petroff (1928) were unable to cultivate filterable forms of the tubercle bacilli from Berkefeld filtrates prepared from pure cultures of these organisms. They also reported that about 33 per cent. of normal guinea pigs had acid-fast organisms in their lymph nodes and expressed the view that the finding of acid-fast organisms in the lymph nodes of animals inoculated with tuberculous filtrates was not due to the regeneration of filterable forms, but merely to the frequent occurrence of acid-fast organisms in normal guinea pigs. Gloyne, Glover and Griffith (1929) also failed to obtain filterable forms of the tubercle bacillus which could produce tuberculosis in guinea

pigs. Wyckoff and Smithburn (1933) studied the development of Mycobacterium phlei on solid media with micromotion photographs. Their micromotion pictures of these organisms in the phase of active growth showed that the bacilli divided by transverse fission but, as the cultures aged, these workers claimed the rod forms fragmented to coccobacillary forms which elongated to small rods of normal bacillary forms, or sometimes they reproduced smaller coccal forms which eventually transformed into rods. Although their findings closely resemble the observations of Kahn (1929, 1930), these authors did not find evidence on the cyclic repeatability of such changes. Nyka and O'Neill (1970) developed a new method of staining Mycobacterium tuberculosis, claiming it to be superior to the conventional methods and, using this new technique, they observed that the shortest and smallest forms of Mycobacterium tuberculosis that could be seen in cultures and tuberculous tissues were the coccobacillary and coccal forms.

The possibility that spirochaetes might have a filterable phase appears to have been the subject of extensive research in the early part of this century. Novy and Knapp (1906) first suggested that the aetiological agent of relapsing fever was capable of passing through Berkefeld filters. Breinl and

Kinghorn (1906) demonstrated that the Berkefeld filtrates of Spirochaeta duttoni were infective to rats. Subsequently Nicoll and Blanc (1914), working on relapsing fever, suggested that the spirochaetes might exist in an invisible, pre-spirochaetal form. According to these workers, it was during the prespirochaetal phase, i.e. when it was going to become visible, that the spirochaetes showed their greatest virulence. Wolbach (1915) conducted studies on the filterability of spirochaetes and came to the conclusion that some organisms larger than ordinary bacteria such as the spirochaetes were capable of passing through Berkefeld V, N and W filters. He found no evidence to suggest that the granules, or the coiled or swollen forms of these spirochaetes were capable of multiplication. Subsequently Nicolle (1927) and Nicolle and Anderson (1929) reaffirmed that the spirochaetes of relapsing fever occurred in two alternating forms in the louse, a virulent invisible form and a visible avirulent form. Nicolle suggested that the spirochaetes fragmented into granular forms which persisted in tissues. The repetitive fever in relapsing fever was brought about by a virulent phase which developed from these granules.

Levaditi, Schoen and Sanchis-Bayarri (1928) described the transformation of Treponema pallidum into granular forms in the testis and lymphatic glands

of injected rabbits. According to these workers, the granular forms resist chemotherapy and persist during the latency period of syphilis and are capable of reverting to the classical forms but they were unable to demonstrate the filterability of these granules. Simon and Mollinedo (1940) made similar observations on Treponema pallidum. De Lamater, Wiggal and Haanes (1950 a & b) studied the development of Treponema pallidum in rabbit testes by stained smears and phase contrast microscopy. They suggested a complex life cycle for this organism, certain stages of which may be related to latency in syphilis. According to these workers the usual mode of reproduction was by means of transverse division but these spirochaetes were capable of producing buds which transformed into 'cyst-like' structures. More recent electron microscopic studies on Treponema pallidum tend to support these cyclical changes, Ovčinnikov and Delektorsky (1971). Levaditi, Vasiman and Chaigneau (1951) studied the life cycle of Borrelia duttoni in developing chick embryos. Their studies suggested the presence of atypical and granular forms of these spirochaetes in organs and tissues of the infected chick embryos. According to them, the granules were capable of transforming to the spirillar form. Klieneberger-Nobel (1951), in her review of filterable forms of bacteria,

suggested that the granular phase of the spirochaetal life cycle may be analogous to the L phase in bacteria which she had first described in 1935.

Studies on the filterable forms in the Salmonella-Shigella- Escherichia coli group of organisms have been carried out quite extensively. Almquist (1911), by filtering typhoid bacilli cultures, obtained small granules which grew with difficulty on ordinary nutrient media at body temperature but which grew luxuriantly on lactose and lactate agar at lower temperatures. These granules were non-pathogenic to guinea-pigs and rabbits but were immunogenic. Friedberger and Meissner (1923) produced pyrexia in guinea pigs by injecting tissues and organs taken from a typhoid case. The guinea pigs did not develop any lesions nor could any organisms be isolated from them. This pyrogenicity was shown to be transferable to other guinea pigs. The filterability of the pyrogenic agent was reported by Friedberger (1927). Gildemeister and Herzberg (1927) were, however, unable to confirm the findings of Friedberger and Meissner. d'Herelle and Hauduroy (1925) had observed that typhoid bacilli, when treated with a bacteriophage, broke up into minute, filterable granules. d'Herelle (1926) subsequently demonstrated that the filterable forms of Salm.typhi, Shigella dysenteriae and B.coli, obtained as a result of the

action of bacteriophages, were capable of producing secondary bacillary colonies. Fejgin (1925 a & b) lysed Salm.typhi with a bacteriophage and injected the filtrates into guinea pigs. Her findings were similar to those of Friedberger and Meissner. Hauduroy (1926 a & b) claimed to have obtained filterable forms of Salm.typhi in polluted water as well as in the blood of typhoid patients. In a series of earlier experiments (Hauduroy 1924 a & b) he had reported that Shigella cultures lysed by bacteriophages and filtered became turbid after prolonged incubation and eventually gave rise to the classical bacillary forms. Bronfenbrenner and Muckenfuss (1927) suggested that the appearance of secondary growth in cultures after filtration through ordinary bacteriological filter candles was due to imperfect filters and faulty filtration techniques. Frobisher (1928) appeared to have repeated some of d'Herelle's and Hauduroy's experiments using different phages of varying potency on a number of organisms. He confirmed the findings of Bronfenbrenner and Muckenfuss and concluded that there was no evidence for the occurrence of filterable forms resulting from the action of bacteriophages on bacteria. He also pointed out that slow-growing contaminants and precipitations in the broth medium could have been responsible for some of the positive filtration

results. Mudd (1928) and Arkwright (1931) had also expressed doubts on the existence of filterable phases in bacteria. It was during this period of conflicting reports on the filterability of bacteria that Hadley, Delves and Klimek (1931) pointed out that the filterable forms found in bacterial cultures are not present at all times but only arise during certain cyclical stages of development of the culture, and that the elements which passed through the candles were special cell forms which, by reason of their morphology and size, were able to pass bacteriological filters. They also pointed out that these filterable phases might demand different methods of cultivation and manifest themselves in an altogether different colonial morphology. Their work is particularly worth mentioning because the G colonies described by them appear to have features in common with the L colonies. These workers produced G type colonies of Shigella by forcing dissociative changes with the help of 0.5 per cent. lithium chloride and pancreatin. Other methods used by them to produce the G colonies were (a) by serial passage in alkaline broth medium, (b) by the use of peritoneal fluid, (c) by the use of bacteriophages, (d) by ageing the cultures, (e) by selection of G colonies from smooth cultures. These enforced dissociative changes produced three types of colonies:- (a) large macroscopically visible

colonies, (b) smaller colonies visible by hand lens and (c) tiny colonies visible only under higher magnifications of the light microscope. After 48 hours' incubation at 37°C the largest of these colonies had a diameter of 0.2 mm (compared with parent S and R cultures which had 2 mm and 4-5 mm diameters respectively). The smallest of the G colonies had a diameter of 0.004 to 0.006 mm. The morphological elements that made up these colonies consisted of coccal bodies occurring in chains or small clusters, granules and thread-like filaments. The filterability of some of these forms was proved. They also showed that G colonies were culturally, morphologically, biochemically and serologically different from the parent culture but it was pointed out that the G cultures could be made to revert to the original form and regain their original characteristics. These workers claimed that G colonies could be demonstrated in E.coli, Salm. paratyphi A & B, Salm.enteritidis, Salm.typhi, Salm.typhimurium and other salmonellae. If their work is examined critically it can be seen that there is convincing evidence to support Klieneberger-Nobel's view (1951) that Hadley and co-workers were, in fact, describing a form intermediary between the bacterial and L phases. Dienes and Weinberger (1951) maintained that they were unable to see any relationship between

the L forms, the filterable phases and the 'G' colonies of Hadley *et al*. Dienes and Weinberger (1951) also raised doubts as to the very existence of filterable forms of bacteria.

Other investigators who have claimed to have obtained or demonstrated filterable phases in some of the common bacterial species were Rosenow and Towne (1917) and Evans and Freeman (1926) workingwith streptococci; Hort (1916) who investigated cerebrospinal fever and meningitis and suggested the existence of filterable forms of meningococci; Lohnis and Smith (1916, 1923) working with *Azotobacter*, *Bacillus subtilis* and *Pseudomonas fluorescens*; Mellon (1926) with *B.fusiformis*; Smith and Jordan (1930) with *Corynebacterium diphtheriae*.

REVIEW OF THE LITERATURE

STREPTOBACILLUS MONILIFORMIS AND L_1 ORGANISMS.

Related to the study of filterable forms was a sudden interest in the Mycoplasma group of organisms. The upsurge of interest in the early 1930's led a number of workers to search for these organisms from various sources to study their aetiological role in various disease conditions. It was during the course of this search that Klieneberger in 1935 discovered L_1 organisms in cultures of Streptobacillus moniliformis. Two years earlier Strangeways (1933) noted that serum broth cultures of Streptobacillus moniliformis, when examined by means of dark ground illumination, showed long branching forms of streptobacilli and amongst these large numbers of spherical bodies of various sizes were present. Many of these spherical bodies contained smaller motile elements. The aggregation of these globoid bodies gave a 'foamy' appearance. These spherical structures, according to Strangeways, did not appear in stained smears but were nevertheless characteristic of serum broth cultures of Streptobacillus moniliformis. Klieneberger's attention was drawn to the reported presence of large swollen round bodies among the filamentous forms of Streptobacillus moniliformis which had a striking resemblance to the Mycoplasma elements with which she had been familiar

earlier. Using a modification of Kuhn's agar-fixation method and dark ground microscopy she studied the colony development of seven strains of Streptobacillus moniliformis (Klieneberger, 1935); also included in her original study was an organism resembling a Streptobacillus which was isolated from the nasopharynx of normal guinea pigs. She observed that serum agar plates inoculated with 24-48 hour serum broth cultures gave rise to colonies of Streptobacillus moniliformis composed of 4 main microscopic elements, namely (a) delicate and fine filamentous structures forming a network, (b) round and oval bodies of various sizes, (c) bacillary chains and (d) in older cultures, granules. Similar elements were also seen in liquid cultures thus confirming Strangeways' earlier observations of the presence of moving particles in the large oval and round bodies. This apparent mixture of bacillary forms and structures resembling PPLO elements led Klieneberger to conclude that a symbiotic relationship existed between two microbes, a pleuropneumonia-like organism and the Streptobacillus. Her initial attempts to separate the symbionts by the usual bacteriological methods were unsuccessful. However, she observed that, subjecting this mixed growth to ageing or heating the cultures to 52-53^{o}C for 5-15 minutes had a more deleterious effect on the bacillary forms and when such treated cultures were

plated out on serum agar plates, tiny pin-point colonies developed between the larger bacillary colonies. These tiny colonies were usually partially embedded in the medium and were characterised by a darker centre and a surrounding lighter periphery, resembling the colonies of pleuropneumonia-like organisms. According to her the growth of these tiny colonies commenced with granules producing a fine network of filaments which, on further incubation, produced round bodies and granules. A 3-4 day old colony thus consisted of a central core of masses of granules and spherical bodies of varying sizes surrounded by a network of filamentous structures. These tiny colonies were completely devoid of bacillary elements. Unsure of the taxonomic status of these new isolates, she designated them as L organisms (L being the abbreviation of Lister Institute). The L isolates from the original seven strains of Streptobacillus moniliformis were designated as L_1 organisms and the PPLO type growth derived from the Streptobacillus isolate obtained from the nasopharynx of guinea pigs was designated as L_2. Although she was able to free the L_1 and L_2 organisms of the bacillary components the converse process was never achieved by her.

In a subsequent paper (Klieneberger, 1936), she described a more suitable medium for the isolation and

maintenance of pure L cultures. She also demonstrated the filterability of these organisms - a property shared by the pleuropneumonia group of organisms. In further morphological studies on L_1 cultures, she once again arrived at her original conclusion that the morphological and cultural properties of L_1 organisms had nothing in common with Streptobacillus moniliformis except that a symbiotic relationship existed between these two organisms. There were, however, some discrepancies in the description of the colonial development of L_1 organisms. Klieneberger and Steabben (1937) obtained isolates closely resembling L_1 organisms from 17 of 19 rats with pulmonary lesions, which were subsequently shown to be immunologically different from the previous L isolates (Klieneberger, 1938) and were identified as L_3 organisms. Klieneberger's observations were so unusual that her findings attracted the attention of contemporary investigatprs working on the PPLO group of organisms. The first confirmation of her observations came from Dienes and Edsall (1937). They were able to get the tiny PPLO type colonies from Streptobacillus moniliformis cultures. The L type growth bred true and was devoid of the bacillary elements. These workers, however, observed that the large bodies arose by direct transformation of bacteria and were capable of independent multiplication.

Dienes and Edsall (1937) appeared to be noncommittal on the question of the nature of the association of the Streptobacillus and the L_1 organisms.

Van Rooyen (1936), in his studies on the biology of Streptobacillus moniliformis, was unable to demonstrate the presence of two independent colonial systems in his cultures, but he did observe many of the morphological variants, including large bodies and granules, described by Klieneberger. He noted that many of the large bodies arose from the enlargement of the bacillary filaments and only a small portion of these bodies remained free or unattached to the filaments. The granules present in the culture were filterable but were non-viable and, according to him, they appeared to have been produced by the bacteria themselves. Dienes (1938) challenged Klieneberger's concept of a symbiotic relationship between L organisms and Streptobacillus. He observed that L organisms appeared in bacterial cultures after 48 hours or more incubation in the form of secondary colonies partly embedded in the growth medium. These, when transplanted initially, appeared to resemble colonial variants of Streptobacillus moniliformis but after the second subculture no bacillary components were present in the L growth on solid agar media. However, when blocks of agar bearing L growth were transferred to nutrient broth, there was initially a pure culture of L organisms

but with further incubation streptobacilli reappeared. He also observed that the original L_1 strains of Klieneberger when similarly transferred into broth medium did not produce streptobacilli. Thus, these marked differences in the variation of the L strains were, according to Dienes, additional evidence against the symbiosis hypothesis. One of Dienes' main considerations against the concept of symbiosis between L_1 and Streptobacillus moniliformis was based on the origin and role of the large forms or bodies found in Streptobacillus moniliformis cultures, but unfortunately his early observations on these forms appear to be both confusing and contradictory. Dienes and Edsall (1937) had stated that the large bodies in Streptobacillus cultures were not degenerative elements but viable forms capable of independent growth in cultures. This statement appears to have been contradicted in a subsequent publication by Dienes (1938) in which he reported that the large forms persist for a while or degenerate and neither in the original cultures nor in the transplants did they show any signs of multiplication. However, he goes on to state in the same communication that the colonies comprised predominantly of large forms and gave rise on subculture to pure cultures of the bacillary forms. Dienes (1939 a & b, 1940 a) made further observations on the colonial development of L_1 organisms. His

observations on the development of the various morphological elements of L_1 organisms differed from the observation of Klieneberger in many respects. He maintained that the properties of L_1 organisms differed much less from those of bacteria than Klieneberger's descriptions had suggested. Dienes pointed out that the development of filaments from granules, as described by Klieneberger, were never observed by him; filaments, large bodies and granules were of bacillary origin. His interpretation of the presence of large bodies was that they arose from the swelling of the bacillary forms and, under appropriate conditions, the large bodies formed typical L colonies. The observation that sometimes the filaments and large bodies produced normal bacilli suggested that the large bodies were not a part of another genetically unrelated colonial system. In support of his view he drew attention to the fact that similar large bodies could be obtained from many other bacterial species under certain conditions, particularly in the presence of calcium or lithium salts. He also reported that not only did the spontaneous development of L type colonies occur in cultures of Streptobacillus moniliformis but that they could also be demonstrated in Haemophilus influenzae, E.coli and a Flavobacterium spp.

Dawson and Hobby (1939 a & b) also reported the

reversion of L_1 organisms into Streptobacillus moniliformis in liquid media. By means of agglutinin-absorption tests, they demonstrated the presence of identical antigens in the L_1 organisms and Streptobacillus moniliformis and, on this basis, concluded that serological closeness suggested that the L_1 organisms were bacillary in origin.

Heilman (1941 a & b) conducted comparative studies on the morphological features and biochemical properties of 12 strains of Asterococcus muris (Streptobacillus moniliformis) and their L_1 derivatives. He arrived at the following conclusions:-

(a) L_1 organisms constituted an avirulent and degraded phase of Streptobacillus moniliformis.

(b) All morphological forms seen in cultures of L_1 were also found in cultures of Streptobacillus moniliformis except the filaments which were absent in the former.

(c) The fine filaments seen in L_1 colonies were the result of distortion arising from stretching or disintegration of thin-walled spherical bodies.

(d) The L_1 organisms were not easily filterable as suggested by Klieneberger.

(e) The fermentative properties of L_1 organisms and Streptobacillus moniliformis were identical.

(f) The growth requirements and biochemical activities of both these organisms were similar.

Smith (1941) also came out against Klieneberger's

hypothesis of symbiosis between L_1 and Streptobacillus moniliformis. He was unable to find any evidence of symbiotic growth in cultures of Streptobacillus moniliformis, nor was he able to show any filterable phases in such cultures. He found that, when wet preparations of these cultures were examined by dark ground illumination under appropriate conditions, isolated groups of bacilli were seen to disintegrate into masses of granules, spheres and large globoid bodies with no trace of the bacillary elements. This was later followed by the reappearance of the bacilli arising directly from the transformation of large bodies. Thus, he supported Dienes' view that the L_1 organisms were a variant phase of Streptobacillus moniliformis. Brown and Nunemaker (1942) were unable to obtain pure L_1 forms directly from broth cultures of Streptobacillus moniliformis. They noted that L_1 colonies were produced only after the initial appearance of the bacillary colonies. These L_1 colonies grew either within the bacillary colonies or isolated from them. When L_1 colonies were transferred to a liquid medium they reverted within two or four days. Klieneberger (1940) held the view that the reversion of L_1 organisms in broth medium was due to the use of insufficiently purified L_1 strains. She suggested that before an L_1 strain could be considered pure it had to undergo 50 to 60 serial passages on solid agar medium. Brown

and Nunemaker (1942), following the method advocated by Klieneberger for purifying the L_1 organisms, found out that, even after 121 serial agar passages, the reversion from L_1 organisms to the bacillary forms took place in broth media. When a culture of Klieneberger's non-reverting strain was subcultured under similar circumstances, reversion to the bacillary forms did not occur. Nevertheless, these authors presented sufficient experimental proof to show that the L_1 - Streptobacillus relationship was one of bacterial variation and not of symbiosis.

Klieneberger (1942) presented new data to justify her classification of L_1 organisms with members of the pleuro-pneumonia group. In this paper she also critically reviewed the experimental evidence presented by investigators who opposed her stand. She stressed the importance of employing proper techniques to study the undistorted morphological constituents of the L_1 colonies. She pointed out that the presence of cholesterol droplets in L_1 and Streptobacillus moniliformis cultures could render the recognition of the genuine elements in these cultures more difficult. Attention was drawn to the dissimilarities in the life cycle of L_1 organisms and bacteria. Klieneberger's views on the morphology and reproductive cycle of L_1 organisms appeared to have been modified. The fundamental reproductive

units were called 'elementary corpuscles' each of which contained a chromatin granule with a small amount of protoplasm. These grew into larger forms with multiple division of the chromatin structure resulting in a large body with multiple chromatin structures. These large forms, with further incubation, segmented into elementary corpuscles. In an earlier paper (Klieneberger, 1940) she had admitted that the fine honeycomb structures produced by filaments were artifacts. She claimed that this mode of reproduction had not been observed in bacteria and that it corresponded in many respects to the development of the pleuropneumonia group of organisms; furthermore, it was pointed out by her that the large bodies found in many bacterial cultures were non-viable, but those found in L_1 cultures produced filterable granules and constituted an important link in the development of these colonies. She also showed by means of cross absorption tests that the L_1 organisms and Streptobacillus moniliformis were antigenically dissimilar. Ørskov (1942) who was familiar with the morphology of pleuropneumonia group of organisms did not accept Klieneberger's interpretation of the close association of these apparently different organisms. According to Ørskov (1942) none of the elements of L_1 colonies of Streptobacillus moniliformis had the slightest resemblance to the pleuropneumonia organisms.

The effect of penicillin in Streptobacillus moniliformis was most striking. Pierce (1942) demonstrated that, while higher concentrations of penicillin were inhibitory to the bacillary forms, the L_1 organisms were unaffected. She was able, therefore, to get pure L_1 colonies by the use of crude penicillin solutions on Streptobacillus moniliformis cultures. The L_1 colonies, however, were capable of reversion to the bacillary state under appropriate conditions. Further cytological studies were carried out by Dienes (1942, 1943, 1946 a & b, 1947 a) and by Dienes and Smith (1942, 1944) on the nature and role of large bodies in the development of L type colonies and also their relationship to the parental bacillary forms. These studies confirmed Dienes' earlier observations that large bodies were produced by swelling of the bacillary elements and that these altered variants were capable of further development in two directions, namely that, under ideal and appropriate conditions they could either (a) produce the elements of the L colonies or (b) reproduce bacteria. These studies provided the unmistakable proof of the true nature and genesis of L colonies that finally convinced Klieneberger-Nobel (1949a) to abandon her symbiosis theory.

TERMINOLOGY

The term "L_1 organisms" was the first descriptive term used by Klieneberger (1935) to describe pleuro-pneumonia-like organisms living in apparent symbiosis with Streptobacillus moniliformis. Her subsequent isolations of similar growth forms from other strains of Streptobacillus moniliformis and other sources were denoted by the letter L followed by a numeral. L stood for Lister Institute and it appears that this designation was originally intended as an interim measure pending further investigations on their exact taxonomic position. Over the years that followed a variety of terms were introduced into the literature by different investigators in this field resulting in considerable confusion and controversy. These terms included L organisms of Klieneberger, L types, L-forms, L phase, L-variants, L-phase variants, L colonies, stable L forms, unstable L forms, L-type bacteria, cell wall defective bacteria, cell wall defective variants, spheroplasts and protoplasts. Dienes and Weinberger (1951) were the first to realise the necessity of using appropriate descriptive terminology to differentiate these altered variants from the colonies they produced. They used the term 'L- form' to denote the elements and 'L- type' for the colonies these elements produced. Basserman et al. (1957)

recommended that the term "L form" in English, 'Formation L' in French and 'L phase' in German be applied to the growth form which arises spontaneously or by stimulation and is characterised on media not containing stimulating substances by colonies having a dense centre and lighter periphery. The elements composing these colonies must not revert to the morphology of the parent culture. The PPLO group of organisms were excluded from this definition. Klieneberger-Nobel (1960) pointed out that the term "L form" was originally intended to denote a type of growth and not the single elements that constituted such a growth. She suggested that the "L form" be used to denote "stable growth which consists of soft protoplasmic elements without defined morphology which no longer possesses rigid bacterial forms nor reverts to them, which can be propagated indefinitely, and which has a characteristic colony on solid medium independent of the bacterial species from which the growth was derived".

Edwards, Klieneberger-Nobel, Dienes, Freundt and Rubio-Huertos (1960) agreed that the term "L phase variant" defined organisms which resembled the PPLO in their morphology but were derived from bacteria under natural or artificial conditions and which were capable of serial propagation irrespective of whether reversion took place or not. It was recommended

that the term "L-form" be restricted to morphological changes only. The terms "unstable L forms" and "stable L forms" have been used by some workers to differentiate the reverting from the non-reverting forms. It was also suggested that the use of these terms be discontinued as all L forms derived from bacteria soon after isolation were capable of reversion and lost their ability to reproduce bacteria only after long cultivation (Dienes, 1968). McGee, Wittler, Gooder and Charache (1971) have dealt with the subject of terminology in a lucid and concise manner. Their definitions of the various forms are as follows:-

Wall defective microbial variants - are microorganisms that have undergone morphological, physiological and cultural changes as a result of damage to or deficiency in their cell walls. These changes do not imply genetic alterations.

L-phase variants - are cell wall defective variants that are capable of serial replication as non-rigid forms and on solid media produce colonies whose central part grows into the medium and in conjunction with its peripheral parts gives the colonies a 'fried egg' appearance.

L-forms - are defined as colonies composed of L-phase variants. McGee et al. (1971) appeared to have reintroduced the terms "unstable", "relatively stable" and "stable" to denote the degree of potential reversion.

Unclassified wall defective variants - are cell wall defective variants which do not conform to the definitions of other types of variants and include predominantly clinical isolates with altered morphology which grow on hypertonic media but cannot be further propagated on the same medium without osmotic supplementation.

Transitional phase variants - are also wall defective variants which have a great tendency to revert to the bacillary phase and microscopically consist of a variety of elements, which vary in shape, size, cell wall constituents, osmotic sensitivity and colonial morphology. They are, however, capable of conversion to the L- phase. Most clinical isolates fall into this group.

Spheroplasts - these generally have most of the features of protoplasts but differ from the latter in that they retain part of the cell wall

materials. Spheroplasts are capable of serial multiplication as spheroplasts but may revert to the bacillary phase or transform to the L phase.

Protoplasts - McGee *et al*. (1971) have defined these as cell wall defective variants completely devoid of cell wall constituents, spherical in shape, osmotically fragile, Gram negative and penicillin-indifferent. These forms maintain metabolic activities and undergo limited divisions.

Brenner *et al*. (1958) recommended that the term protoplast be applied only to globular or spherical forms of altered bacterial variants in which it has been established that cell wall structures are completely absent as determined by the absence of (i) cell wall antigens, (ii) chemical components associated exclusively with the cell wall and (iii) phage receptor sites as well as being osmotically sensitive and assuming a spherical shape in suitable media. It should be shown by electron microscopy that a cell wall is lacking.

MORPHOLOGY OF L COLONIES

On solid media.

On suitable solid media L form growth is characterised by extremely small rounded colonies having a dark centre surrounded by a lighter periphery. The appearance of the dark centre is due to the growth of the colonies into the agar medium. Such colonies, when examined under low magnifications of the light microscope, show a granular centre with a foamy periphery; this colonial morphology resembles very closely that of the colonies produced by Mycoplasma. Madoff (1960) reported that L colonies could be distinguished from the Mycoplasma colonies by size and staining properties. L colonies when fully developed were much coarser in appearance, larger in size and stained more intensely than Mycoplasma colonies. The morphology of L form colonies is similar in appearance irrespective of the species from which they are derived (Dienes, 1939b; Freundt, 1950). The effect of penicillin in producing L transformation in various species of bacteria has been studied by Dienes (1949a), and by Dienes and Weinberger (1951). Proteus cultures on media containing penicillin produce two types of L colonies. These were designated by Dienes (1949a) as Types 3A and 3B colonies. Type 3A colonies were

always small, rarely exceeding 0.3mm, whereas type 3B colonies always grew within the agar and were larger, measuring 2mm or more when fully developed. Both these types had the characteristic 'fried egg' appearance except that the 3B type had a broader peripheral zone. The structural elements in both colony types appeared to be similar when viewed under higher magnifications of the light microscope. Types 3A and 3B, however, differed markedly in their nutritional requirements and their ability to revert to the bacillary state. The growth of L colonies into the agar and the ultimate appearance of these colonies on solid media were reported to be due to the very small size of the reproductive bodies, the plasticity of the elements that constituted these colonies and the physical properties of the medium on which they were grown (Razin and Oliver, 1961; Dienes and Madoff, 1968; Dienes and Bullivant, 1967; Dienes, 1968; Hijmans, van Boven and Clasener, 1969). Razin and Oliver (1961) who studied the morphogenesis of L colonies, found that under normal conditions when L forms were plated on solid media, growth of these forms began within the media due to the drawing in of the tiny reproductive L elements into the interfibrillary spaces of the agar as a result of capillary action. Such physical forces were exerted only when the surface of the agar medium was dry. With moist agar surfaces

the imbibing properties of the agar were less, and consequently the reproductive elements of the L colonies were not subjected to the same physical forces. Due to the absence of multiplication of these elements within the agar, the typical colonies with a dark centre and a lighter periphery were not produced. The width of the periphery of L colonies was also shown to be determined by the amount of free moisture present on the surface of the agar medium, so that in the presence of a thin film of moisture, large peripheral zones resulted, while decreasing the amount of free moisture by drying the agar plates resulted in narrow peripheral zones. The effect of agar concentration was also shown by these workers to alter the morphological appearance of L growth. With very low agar concentrations L colonies of Streptobacillus moniliformis grew as irregularly round colonies at various depths of the medium with no differentiation of central and peripheral zones and a hazy surface growth was also present. With increased concentrations of agar, wider peripheral zones were formed up to an optimal concentration of agar beyond which further increases in concentration had the converse effect, not only of reducing the size of the peripheral zone but also of decreasing the central zone diameter. Dienes and Bullivant (1967) and Dienes (1968) tend to support the view

that the granules of L colonies actively penetrate the agar to produce the central submerged growth. They also pointed out that on solid media which do not permit active penetration, the L elements do not divide but merely increase in size.

Kandler and Kandler (1956) and Kandler and Zehender (1957) described a type of growth having the characteristics of the 3A type but in which the individual elements were completely lacking in the cell wall constituent diaminopimelic acid. These workers thought this type was of mutational origin.

Surface growth of L colonies without agar penetration has been reported in some Escherichia coli strains (Dienes & Sharp, 1956; Schumann & Taubeneck, 1969; Seeberg, 1973; Seeberg & Brorson, 1974).

The ability of some gram negative bacteria to produce homogeneous L colonies lacking the characteristic appearance was shown not only to be dependent on cultural conditions but also to be due to the inherent properties of some strains. Seeberg & Brorson (1974) studied L colony production and formation in 19 E.coli strains and found that several factors influenced the production and morphology of the L colonies. The strains of E.coli with which they worked produced two types of growth under the influence of varying concentrations of antibiotics on media with pH of 5.0, 6.0 and 7.0. Eighteen of the

19 strains produced L colonies. The L growth was manifested in two forms:-

(a) the classical L type growth with a 'fried egg' appearance,

(b) the homogeneous L type growth lacking this classical appearance.

The latter types of growth occurred over a wide pH range and concentration of antibiotics. It appears from their work that the development of homogeneous L type growth tend to be favoured by certain methods of cultivation especially in particular strains of E.coli. Prolonged cultivation of Type 3A and Type 3B colonies on agar media bring about morphological changes in the L growth particularly those derived from Proteus and Salmonella spp. (Dienes, 1970b). These colonies were able to grow on the surface of agar with little or no penetration into the medium and consequently the usual dark centres were absent; in these cases the L growth resembled more closely the bacillary type of growth.

Three types of L colonies appeared to have been obtained from a single strain of Pseudomonas aeruginosa under the influence of carbenicillin (Bertolani, Elberg and Ralston, 1975). Obtained under identical conditions, the three differed in their rate of growth and the diameter of the central dark core.

The first type was virtually devoid of a central core, the second type had a small core and the third type had the largest central zone denoting the degree and extent of agar penetration by the L elements. L colonies of the various serotypes of Listeria monocytogenes showed similar core size variations (Brem & Eveland, 1968a).

Various other atypical L growths have been described. Pachas and Currid (1974) found that in related strains of Erysipelothrix rhusiopathiae while the parent strain produced typical L colonies, under similar conditions, the revertant L strains of the same strain produced spontaneous L colonies having a heavy central growth with a deep and narrow peripheral growth. The mechanism of development of these 2 types of growth was also different. Clostridium perfringens as a group is known to produce atypical L colony variants. Fully developed L colonies of Cl.perfringens, Types A and D occurred as two forms (Kawatomari, 1958). One had a convex surface with the submerged part of the colonies diffused into the agar without demarcation, whereas the other type was flat with well defined radiating growth. Cl.perfringens types B and C produced a third type of L colony variant which appeared as a large colony with numerous secondary colonies within it. L colonies of Cl.perfringens E type were the smallest

L colonies of the Cl.perfringens group and were compact and domed and resembled α-haemolytic streptococci (Kawatomari, 1958).

In liquid media.

In liquid media the original L_1 organisms derived from Streptobacillus moniliformis grew as clumps attached to the sides of the vessels in which they were cultivated or as heavy flakes (Klieneberger, 1936; Klieneberger-Nobel, 1960; 1962; Heilman, 1941a). Initially L form growth in liquid medium appeared as granular deposits at the bottom of the medium with a clear supernatant, but the passage of these forms in liquid media often resulted in the formation of a viscous and mucoid type of growth (Hijmans et al., 1969). Slimy growth in liquid media is characteristic of staphylococcal L forms (Marston, 1968) but turbid non-slimy growth has also been recorded. Dienes (1967a) reported that it was not easy to obtain good growth of L forms in liquid media, but old L form strains grown in broth media produced mucoid masses which formed a shallow layer in flasks containing such media. L form broth cultures of Erysipelothrix rhusiopathiae produced heavy sedimentation in broth consisting of clumps and granules, but no turbidity of the broth occurred even after agitation of the culture (Pachas and Currid, 1974). Gilpin, Young and

Chatterjee (1973) were able by selection to obtain a stable L form of Bacillus subtilis which grew in broth without producing clumps. Homogeneous broth cultures of L forms of streptococci were obtained by serial passages of these forms in liquid media (Panos and Barkulis, 1959). Broth cultures of Proteus L forms were obtained from strains that grew on the surface of liquid media as a thick film or pellicle (Dienes, 1953a; Tulasne, 1950). This type of growth is the result of permanent structural changes in the L elements (Dienes, 1968). L forms of Vibrio spp. have also been observed to produce a similar type of growth in liquid media (Minck & Lavillaureix, 1956).

THE MICROSCOPIC MORPHOLOGY OF 'L' ELEMENTS

The usual bacteriological techniques for the demonstration of microorganisms in bacterial cultures are unsuitable for the study of the allegedly soft and friable bodies that constitute the L colonies. These elements that lack a cell wall either in part or in toto have cell walls that have been structurally modified, tend to become completely unrecognisable during the conventional methods of drying and fixing of the smears (Klieneberger-Nobel, 1960; Dienes, 1970c). Other factors that impede the proper observation of the microscopic elements is the growth of some of these bodies within the medium and therefore techniques used to demonstrate these forms have to be such as to cause minimal distortion. Many of the early descriptions of these elements were incomplete or lacked accuracy. The occurrence of oily droplets in Streptobacillus moniliformis L cultures grown on media containing serum was reported by Partridge and Klieneberger (1941). The condensation of these oily globules had often made interpretation of L colony elements difficult (Klieneberger, 1942). In Klieneberger's publications between 1935 and 1951 there are fundamental differences in her descriptions of the microscopical elements that made up the L colonies. Some of the structures originally thought

by her to be developmental forms were shown ultimately to be nothing more than artifacts (Klieneberger, 1940). Likewise, the early observations of Dienes on the constituents of the L colonies were also far from complete. On the basis of the extensive microscopical studies of Klieneberger (1942), Klieneberger-Nobel (1951, 1960, 1962), Dienes and Weinberger (1951), Dienes (1967a, 1968), Dienes and Bullivant (1967) and Dienes and Madoff (1968) the following microscopic elements have been recognised in pure L colonies:-

Large bodies - these arise from the swelling of the bacilli and are generally spherical in shape in liquid media and irregularly round or polygonal on solid media. They are reported to be fragile, pliable and also osmotically sensitive. They may be vacuolated, contain granules or even bacilli. They measure from 1 μm - 50 μm in diameter and occur mostly around the more peripheral parts of the colony.

Granules - occur in heaps mainly in the central area and also grow into the media. They measure between 0.5 μm to 1.0 μm in diameter (Hijmans et al., 1969) and occur both within the large bodies or as extracellular aggregates. They may be spherical,

elongated or even irregular in shape but are much smaller than bacteria Dienes, 1968). Klieneberger (1942) and Klieneberger-Nobel (1962) tends to regard them as spherical and, according to her, these bodies occur as densely staining elements that lie in a matrix. Swollen granules measure between 2-3 mμ in size and whether these are the same as coccal elements described by Klieneberger-Nobel (1960) is uncertain.

Elementary corpuscles - these appear to be the smallest of the L elements and measure between 0.05μm to 0.5 μm (Hijmans et al., 1969). These bodies appear to be the same structures referred to by Klieneberger-Nobel (1951) as filter-passing elements. She estimated their size to be between 175-250 nm. Subsequently she had stated that the smallest of the L elements have a diameter ranging from 200-300 nm (Klieneberger-Nobel, 1962). It would appear that the largest of these elements would lie just within the limits of resolution of the ordinary light microscope.

> The smallest forms seen by light microscopy ranged in size between 0.3-0.5 μm (Dienes & Weinberger, 1951). They are regular in shape and in young colonies occur at the edges.

The foregoing descriptions of the morphological elements generally apply to L form elements that have been examined immediately or shortly after L induction. It is now known that gross morphological alteration can take place in L forms of Proteus spp. and Salmonella spp. due to prolonged serial transfers from one medium to another (Dienes, 1970b & c). The altered colonial variants consisted of large bodies (75μm), small round forms (1-3μm), granules (<1μm) and filaments. Two types of large bodies have been observed. The first type, similar to those found in 3A and 3B type L colonies, occurred sparsely and stained less intensely. The second type which occurred more profusely was much smaller in size and was generally resistant to distortion. In young cultures the large bodies assumed different shapes and a variety of intermediate types occurred between these and the small round forms. The small round forms were also very resistant to distortion and stained intensely. The filamentous forms stained poorly and showed true branching. Some of these filaments and their branches terminated in granules,

knobs or spherical bodies. The colonies usually consisted of a mixture of elements but cultures with one or more of the predominant elements occurred.

The morphological elements found in solid media also occur in liquid media. In the latter, large bodies tend to be spherical and occur in various sizes up to 50 μm in diameter. Most of the large bodies show varying degrees of vacuolation though some may appear non-vacuolated or may even contain granules (Dienes, 1967a). The presence of filter-passing elements in L form broth cultures has been recorded by Klieneberger-Nobel (1949b). Minck and Lavillaureix (1956) found, in addition, 'dwarf' forms and large numbers of filaments. Their observations do not appear to have been confirmed although Weibull and Lundin (1963) observed thread-like structures connected to spherical bodies. Filamentous forms with terminal granules or spherical swellings have been observed in broth cultures of Salmonella and Proteus spp. which have acquired permanent alterations (Dienes, 1970c). Altenbern and Landman (1960) grew Proteus mirabilis mutants in liquid medium. The L bodies of these were predominantly multinucleate.

INDUCTION OF L PHASE VARIANTS

Several Gram negative and some Gram positive bacteria produce L type growth under ordinary cultural conditions. The natural occurrence of this type of growth has been observed in cultures of Streptobacillus moniliformis, Fusiformis necrophorus, Haemophilus parainfluenzae, Flavobacterium spp. Neisseria gonorrhoea, Vibrio cholerae and the viridans group of streptococci Bacillus subtilis and Bacteroides ruminicola (Klieneberger, 1935; Dienes, 1939a & b, 1940a, 1941, 1942, 1946a &b; Carrère & Roux, 1953a; Hijmans & Dienes, 1955; Pachas & Dienes, 1968; Madoff, Burke & Dienes, 1967; Cheng, 1973). Transformation from the bacillary to the L phase has been shown to be related to pleomorphism and the presence of large bodies (Dienes, 1942, 1946a & b). Bacteria or their filaments in some cultures swell up into large bodies and these eventually produce either L type or bacillary growth. In cultures of Streptobacillus moniliformis and Fusiformis necrophorus very often most of the bacilli swell up to produce large bodies, hence the ease with which L colonies are formed. While the production of occasional large bodies occurs in most bacterial cultures, it is only when large bodies are produced en masse that L type growth is manifested. Such a mass transformation under normal conditions is a property of some species

and very often is strain dependent. This property of transformation is generally lost after a few passages in broth. Cultures that produce these abnormal forms tend to undergo autolysis. Dienes (1946a, 1970c) considers that the spontaneous transformation to large bodies and their further development into L form colonies result from the combination of various factors such as the diffusion of metabolic products into the medium, the autolysis of the bacillary forms and the suitability of the medium itself.

The production of L forms by artificial methods basically involves three factors:-

(a) Induction of morphological or structural defects in the cell wall. This may be achieved by partial or complete removal of the cell wall by cell wall destroying enzymes or by the prevention of synthesis of cell wall components that give rigidity to the bacterial cell,

(b) The prevention of lysis of these wall defective variants, and

(c) The provision of suitable substrates and environmental conditions for the multiplication of these forms.

Induced transformation of bacteria into L forms arose as a result of Pierce's observation (1942) that L forms were unaffected by concentrations of penicillin which inhibited the bacillary forms of Streptobacillus moniliformis. This selective action of penicillin

enabled Pierce to obtain pure growth of L colonies from the bacillary forms. Following these observations Dienes used penicillin to induce L colonies in a number of gram positive and gram negative bacteria (Dienes, 1947a, 1948a & b, 1949; Dienes and Weinberger, 1951). Semisynthetic penicillin and the closely related cephalosporins were particularly useful for the production of L forms from penicillin-resistant organisms (Kagan, Molander & Weinberger, 1962; Chang & Weinstein, 1964; Hamburger & Carleton, 1966a; Watanakunakorn & Hamburger, 1969; Watanakunakorn, Goldberg, Carleton & Hamburger, 1969a; Hamburger & Carleton, 1968; Abbate, Leonessa & Altucci, 1973; Bertolani et al., 1975; Roberts, 1968; Marston, 1968).

The relationship between antibiotic sensitivity and capacity to produce L forms is best illustrated in Pseudomonas aeruginosa and Staphylococcus spp., Braude, Siemienski and Lee (1968) were unable to induce spheroplasts in Pseudomonas aeruginosa with benzyl penicillin. Chang and Weinstein (1964), while being able to produce morphological changes of several gram negative organisms using different concentrations of cephalothin, were unable to induce similar changes in Pseudomonas aeruginosa.

Carbenicillin, which is known to be moderately active against Pseudomonas aeruginosa, used in high

concentrations produced L growth from this organism (Watanakunakorn & Hamburger, 1969; Hubert, Potter, Hensley, Cohen, Kalmanson & Guze, 1971; Bertolani *et al.*, 1975). Methicillin was likewise particularly effective in producing L forms from penicillin-resistant and methicillin-resistant staphylococci (Kagan *et al.*, 1962). Differences have been observed between natural and synthetic penicillins and cephalosporins in the ability of bring about induced L transformations; penicillin induced L colonies in *E.coli* over a narrow range of concentrations whereas synthetic penicillin and cephalothin did so over a much wider range (Seeberg & Brorson, 1974).

The mechanism by which penicillin causes the bacterial cells to lose their rigidity has been reported by Park and Strominger (1957), Park (1968) and Strominger (1968). Penicillin acts by interfering in the biosynthesis of cell wall murien. In cell wall synthesis there is a complex sequence of events which lead to murien synthesis. Penicillin inhibits the transpeptidation of D-alanyl-D-alanine, the last stage of cell wall synthesis (Tipper & Strominger, 1965; Wise & Park, 1965). The site of action of cephalosporins is similar to that of penicillins. Thus in both the penicillin group and the cephalosporins, the bacteria continue to produce uncross-linked or defective murien and, in combination with the lytic

enzymes, convert bacterial cells to spheroplasts (Strominger, 1968).

D-cycloserine is a structural analogue of D-alanine and inhibits the formation of D-alanyl-D-alanine, thus in contrast to penicillin it acts on the first stage of cell wall synthesis. This inhibitory action is competitive and can be reversed by D-alanine (Neuhaus & Lynch, 1964; Richmond, 1966; Strominger, Izaki, Matsuhasi & Tipper, 1967). D-cycloserine has been used for the induction of L type growth in Group A β-haemolytic streptococci, Staphylococcus aureus, Proteus rettgeri, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella spp. and Mycobacterium tuberculosis (Michel & Hijmans, 1960 Ward & Martin, 1962; Krawitt & Ward, 1963; Garwin & Mattman, 1967; Watanakunakorn, 1971a). L forms were also obtained from a number of pathogenic Neisseria by the use of this antibiotic (Roberts, 1968).

Although novobiocin has been shown to intefere with cell wall formation (Strominger & Threnn, 1959), a number of investigators have failed to induce L forms with this antibiotic (Krawitt & Ward, 1963; Molander, Kagan, Weinberger, Heimlich & Busser, 1964; Rotta, Karakawa & Krause, 1965; Roberts, 1968). Molander and associates (1964) suggested that the failure of novobiocin to induce L forms was because of the action of the drug on the cytoplasmic membrane. Rotta

et al.(1965) ascribed this failure to the intracellular deficiency of magnesium ions in treated organisms. Their views are based on the findings of Brock (1962) that novobiocin binds magnesium ions possibly leading to an intracellular deficiency of these ions. Gooder (1968a) has claimed that L forms could be induced by novobiocin but no experimental details of this were given.

Bacitracins and polymixins are members of the peptide group of antibiotics. Both these have been used with varying degrees of success to induce L growth from a number of bacterial species. Initial attempts to induce L growth with bacitracin were unsuccessful (Ward, Madoff & Dienes, 1958; Krawitt & Ward, 1963; Hancock & Fitz-James, 1964). However, Krawitt and Ward (1963) as well as Hancock and Fitz-James (1964) were able to induce protoplast,formation in Proteus spp. and Bacillus megaterium respectively. Subsequent reports have indicated that bacitracins act as L transforming agents in some strains of streptococci as well as in strains of Neisseria (Rotta et al., 1965; Roberts, 1968). These observations are particularly interesting in view of the fact that bacitracin is not only affects cell wall synthesis at the level of the cytoplasmic membrane but is also very active against protoplasts (Garrod & O'Grady, 1971).

Antibiotics of the polymixin group act on the

cytoplasmic membrane (Kagan, 1968) and this it would not be expected to be an L inducing agent. As far as is known, only Edman and Mattman (1961) have used polymixin B to induce subsurface growth of L colonies of Pseudomonas aeruginosa in the presence of 20 per cent. horse serum.

Vancomycin is a large molecule antibiotic which interferes with cell wall formation in the second stage of the biosynthetic process by causing an accumulation of hexosamine containing uridine nucleotides (Strominger, 1968). It has been reported that this antibiotic does not readily induce L transformation (Krawitt & Ward, 1963; Hancock & Fitz-James, 1964; Williams, 1963; Strominger, 1968). Hancock and Fitz-James (1964) have presented evidence that the lysis and the failure of Bacillus megaterium protoplasts to grow in the presence of vancomycin are due to the direct action of this antibiotic on the cytoplasmic membrane. These workers suggested that the accumulation of cell wall precursors in the bacteria was a secondary effect arising from the consequence of cytoplasmic membrane damage. Roberts (1967, 1968) has claimed the induction of L forms in Group B Neisseria meningitidis by the use of this antibiotic. L forms of Staph.aureus have also been induced recently by vancomycin (Watanakunakorn, 1971b). Ristocetin shares many similarities with vancomycin in its pharmacological action, in its spectrum

of activity (Garrod & O'Grady, 1971), in the mechanism of interference in the biosynthesis of cell wall and in the induction of cell wall defective variants (Strominger, 1968). This antibiotic, like vancomycin, has only been reported to induce L colonies in Group B Neisseria meningitidis (Roberts, 1967).

Antibiotics such as kanamycin, neomycin, streptomycin, gentamycin, chloramphenicol, tetracycline and the macrolide group of antibiotics have generally failed to induce L growth in vitro. The only reports of L form induction from this group of antibiotics are by Dienes, Weinberger and Madoff (1950a) who obtained arrested L colony forms in Salmonella typhosa and Salm. typhimurium exposed to aureomycin and chloramphenicol and by Edman and Mattman (1961) in a brief communication that dihydro-streptomycin could induce L colony growth in Pseudomonas aeruginosa.

Bacteriocins have been reported recently to be capable of inducing L growth in Clostridium perfringens (Mahony, Butler & Lewis, 1971). They observed that bacteriocin was only effective in inducing L growth in metabolically active cells. Their studies indicated that this agent does not block the synthesis of RNA, DNA or protein and thus its exact mechanism of action in inducing L transformation is yet to be elucidated.

A considerable amount of literature has accumulated on the use of muralytic enzymes to produce bacterial protoplasts and L forms since Weibull's (1953) demonstration of the action of lysozyme on Bacillus megaterium in an hypertonic medium. The muralytic enzymes that have been known to induce L type growth are lysozyme, a phage-associated lysin and lysostaphin. Fremier, Krause and McCarty (1959) obtained L growth from Group A streptococci which were treated with phage-associated lysin obtained from the lysate of Group C streptococci. Gooder and Maxted (1961) have made similar observations.

L forms have been induced by exposure of Bacillus subtilis and Groups A and D streptococci to lysozyme in an hypertonic milieu. When the protoplasts thus obtained were transferred to solid media they grew as L colonies (Landman & Halle, 1963; King & Gooder, 1965, 1970a; Madoff, Burke & Dienes, 1967). In Bacillus anthracis transformation into spheroplasts occurred only in very high concentrations of lysozyme with CO_2-supplemented incubation (Chatterjee & Williams, 1965). Both these muralytic enzymes act by depolarisation of the existing murein.

Another enzyme system that has been used recently for L growth induction is lysostaphin. Lysostaphin was first described by Schindler and Schuhardt (1964) as a bacteriolytic factor which had the unique property

of lysing staphylococci but not other bacteria. When staphylococci were exposed to lysostaphin the removal of cell wall was rapidly accomplished (Browder, Zygmunt, Young & Tavormina, 1965).

Lysostaphin has been used successfully for the induction of L colonies only from staphylococci (Watanakunakorn, Goldberg, Carleton & Hamburger, 1969b; Marston, 1968).

Amino acids have been used to induce L growth in Salmonella typhi, Salmonella typhimurium, Haemophilus influenzae and streptococci, (Dienes, Weinberger & Madoff, 1950a; Dienes & Zamecnik, 1952; Madoff, 1970; Diena, Wallace & Greenberg, 1964; Lapinski & Flakas, 1967; Want & May, 1975). Of the amino acids that have been used, glycine appears to be the most effective L inducer (Dienes & Zamecnik, 1952). The D-amino acids and glycine induce L transformations by causing the reversal of the terminal cross-linking reaction in cell wall synthesis (Strominger, 1968).

The lysis of bacteria by a specific antibody-complement reaction is well known. Bacteria when exposed to such a system and subsequently treated with a lysozyme produce spheroplasts (Muschel, 1968, Davis, Gemsa & Weidgwood, 1966). Viable spheroplasts can also be produced by exposing bacteria to the action of antibody and complement without post-treatment with lysozyme in an osmotically protected environment

(Freeman, Musteikis & Burrows, 1963). However, the ability of these forms to replicate as L colonies on solid media has only been demonstrated in Salmonella typhi, Listeria monocytogenes and Vibrio cholerae (Dienes et al., 1950a; Iannetta & Wedgwood, 1967; Brem & Eveland, 1968a). It has been suggested that the production of spheroplasts by the combined action of antibody, complement and lysozyme is the result of complement causing damage to the cytoplasmic membrane of the bacterial cell, thus indirectly disrupting cell wall synthesis, and the subsequent dissolving action of lysozyme on the already present cell wall mucocomplex (Muschel, 1968).

The additive effect of two different inducing agents such as glycine and the penicillins or similar antibiotics in producing L colonies has been reported by a number of investigators (Michel & Hijmans, 1960; Madoff et al., 1967; Madoff, 1970; Want & May, 1975). Brucella L forms induced by combined penicillin-glycine treatment have been maintained on glycine medium without reversion, but withdrawal of any of the transforming agents from the medium resulted in reconversion to the bacillary phase (Christoforov & Peshkov, 1969). Lapinski and Flakas (1967) were also able to obtain L growth of H.influenzae with these combined agents but they were

unable to increase the yield of L colonies on a glycine induction medium without the subsequent addition ofpenicillin. Fedorova (1965) reported that glycine-induced Salm.typhimurium spheroplasts were only capable of forming L colonies on a hypertonic medium containing penicillin. Nothing much can be inferred from this author's results as cultures of the same organism were able to produce L colonies in the presence of penicillin alone in a similar type of medium without added glycine.

Chatterjee and Williams (1965) reported the dual effect of combined lysozyme and glycine in producing B.anthracis spheroplasts. Glycine alone was incapable of producing spheroplasts. Lysozyme by itself, although it was able to produce the same effects as in the combined treatment, had to be used in very high concentrations (ten times that used in the combined treatment). Brem and Eveland (1967) found that glycine-lysozyme treatment, though effective in producing L growth, was inferior to penicillin in bringing about similar transformations.

Ethylenediamine tetra-acetic acid (EDTA), a powerful chelating agent, and lysozyme have been used for the production of Mycobacterium tuberculosis spheroplasts. These, when transferred to a solid medium under appropriate conditions, grew as L colonies (Willett & Thacore, 1966). Spicer and

Spooner (1974), using the same agents, were unable to get any viable E.coli spheroplasts. The L phase of Group A streptococci has also been induced by infection of the bacteria with virulent phages (Maxted, 1968). A number of other incitants and transforming agents have been used to procure L growth in various species of bacteria; included amongst these were sulphonamides in N.gonorrhoeae (Brown & Hayes, 1942), carboxymethylamine in Salm.typhi and Salm.typhimurium (Dienes et al., 1950a; Dienes & Weinberger, 1951), crystal violet, radioactive sodium, sulphur and fresh serum in several species of Salmonella (Mattman, Tunstall & Kispert, 1969), serum in Corynebacterium spp. (Poetschke, 1955), anti-metabolites in clostridia (Dienes, 1950), glycocoll in Brucella spp. (Christoforov & Peshkov, 1969), sucrose in Clostridium perfringens (Mahony et al., 1971), streptomycin deprivation in streptomycin-dependent strains of Salm.paratyphi (Landman & Burchard, 1962) and nutritionally poor media in staphylococci (Mattman, Tunstall & Rossmoore, 1961). Ultraviolet radiation has been reported to cause L transformations in Agrobacterium tumefaciens (Rubio-Huertos & Cabezas de Herrera, 1966).

Normal mammalian sera have been shown to convert viable E.coli and rough strains of Salm.typhosa in media containing stabilising agents, electrolytes and Mg^{++} (Muschel, Carey & Baron, 1959). Smooth strains

of Salmonella typhosa remained unchanged by normal sera, but in the presence of antisera, complement and lysozyme were transformed into stabilised protoplasts.

FACTORS AFFECTING INDUCTION, YIELD AND PROPAGATION OF L FORMS.

The transition of a microorganism from a bacterial state to an L-phase is usually accompanied by marked changes in the growth requirements arising mainly as a result of modifications to or the physical loss of the rigid cell wall in which a number of biochemical and physiological processes are segregated.

Osmotic stabilisers.

The extent of a volume increase or shrinkage of a bacterial cell consequent upon differences in the osmotic pressure within it and its immediate environment are limited within the narrow limits of the elasticity of the cell wall. Thus volume regulation becomes a serious problem in wall-less bacteria. Failure to provide osmotic protection to wall-less forms will lead to lysis of the cell, in a hypotonic environment.

Weibull (1953) was the first to demonstrate that osmotic lysis could be prevented in protoplasts prepared with lysozyme by increasing the osmolarity of the suspending medium. One of the most important findings in the study of bacterial L forms was the discovery by Sharp (1954) that a high concentration of salt in the medium was necessary for the induction and propagation of L forms of some bacterial species. Even in species that readily gave rise to L type growth, transformation

into L colonies was more easily achieved on solidified agar media than in liquid media. In most instances primary growth of L forms in ordinary liquid media has been unsuccessful. These observations suggested that some sort of support, physical or functional, akin to the cell wall was necessary before they could express themselves as viable entities capable of propagation. A number of substances are available to provide osmostabilisation to protoplasts, spheroplasts and other wall defective variants. Among the substances that have been used as primary osmotic stabilisers were sodium chloride, sodium lactate, sodium succinate, ammonium chloride, polyethylene glycol, polyvinylprilidone and sucrose. In a number of induction media, salts intended as nutritional supplements or as buffers provide significant osmolarity to the medium. The nature of the osmotic stabiliser and the concentrations necessary for L induction and propagation vary in different species of bacteria. No special osmotic stabilisation was required for the first recorded isolation of L forms from Streptobacillus moniliformis (Klieneberger, 1935) nor for many of the L forms derived from a number of gram negative bacteria (Dienes & Weinberger, 1951), whereas a large number of gram positive cocci required high salt concentrations (Sharp, 1954; Dienes & Sharp 1956; Crawford, Frank & Sullivan, 1958; Williams

1963; Madoff et al., 1967; Marston, 1968). The wide differences in the osmotic pressure exerted by the cell contents in Gram positive and Gram negative bacteria led Hijmans and associates (1969) to suggest that L induction in Gram negative species can be achieved on media with the usual sodium chloride concentration whereas in Gram positive bacteria, consistent induction into L forms could generally be achieved only with increased salt concentration in the induction media. Apart from the interspecies difference in the requirement for osmostabilisation, a number of observations indicate that strains within a species vary widely in their need for osmotic protection. Different strains of Group A beta haemolytic streptococci required varying concentrations of sodium chloride for L induction (Sharp, 1954). In the alpha haemolytic streptococci the sodium chloride requirements within the strains varied more widely - some were induced with no added salt (Dienes, 1953b) while the majority of these required high salt concentrations (Dienes & Sharp, 1956). Protoplasts derived from different strains of B.megaterium varied in their osmotic requirements (Weibull, 1958). L forms derived from a number of bacterial species which initially required high salt concentrations have been adapted to grow at lower concentrations of salt (Williams, 1963; Marston, 1968; Madoff, 1970;

Gilpin et al., 1973; Clasener, Ensering & Hijmans 1970). Sucrose has been widely used to provide osmotic protection in induced transformations. The concentrations used to provide adequate protection in the most vulnerable of the wall defective variants varied from 0.1M (Weibull, 1953) to 1.2M (Mitchell & Moyle, 1956; 1957; Palkins & Fikhman, 1964). The concentrations of sucrose that have been reported effective in stabilising L phases were 1.0M to 1.2M for L phases of Gram positive cocci (Dannis & Marston, 1965; Maxted, 1968) and between 0.3M to 0.54M for Gram negative species (Lederberg and St.Clair, 1958; Landman, Altenbern & Ginoza, 1958; Roberts, 1968; Seeberg & Brorson (1974). L forms derived from some strains of Streptococcus MG have the ability to adapt themselves to grow in media where the sucrose concentrations have been gradually reduced (Madoff, 1970) and in Streptococcus faecalis the L forms have been gradually adapted to grow in the absence of sucrose or any other osmotic stabilisers (Montgomerie, Kalmanson & Guze, 1972). In a number of bacterial species the nature of osmostabilisers appears to be a critical factor in the induction of L forms. In pneumococci and some strains of Streptococcus MG, L forms were not induced by high salt concentrations but, when the required osmolarity was provided by sucrose, L forms were readily formed (Madoff & Dienes,

1958; Madoff, 1970). In Neisseria gonorrhoea L induction occurred when sucrose was used as the osmotic stabiliser but not with sodium chloride (Dienes, Bandur & Madoff, 1964). For the induction of L forms in the acid-fast organisms, sucrose as a stabiliser was superior to high sodium chloride concentration (Mattman, Tunstall, Mathews & Gordon, 1960). Substitution of sucrose with polyvinylpyrilidone in solid agar was without effect on the induction or reversion of L forms of Ps.aeruginosa (Bertolani et al., 1975). A high solute concentration provided by potassium phosphate and potassium succinate did not produce L colonies but with sucrose, high yields were obtained (Landman et al., 1958). Multiplication of B.megaterium protoplasts was not possible with sodium chloride but was so with sucrose or succinates (Kusaka, 1967). Nimmo and Blazevic (1969) found that sucrose was superior to sodium chloride as a stabilising agent in L forms derived from several bacteria. Even in bacteria which produced L colonies on media containing high salt concentrations the choice of the salt was important, thus β-haemolytic streptococci which produced L growth on high sodium chloride agar media differed in their ability to be induced on media containing other salts such as disodium hydrogen phosphate or calcium chloride (Dienes & Sharp, 1956). On the other hand, Minck and

Lavillaureix (1956) considered that the nature of the salt used was unimportant as long as they provided an hypertonic environment conducive for transformation. Landman and Ginoza (1961) demonstrated the interchangability of sodium chloride with sodium succinate or potassium chloride to provide the tonicity in the medium to support L forms of Salm.paratyphi. Montgomeri, Kalmanson & Guze (1967) reported that sucrose as well as sodium chloride used as osmotic stabilisers supported the growth of Streptococcus faecalis protoplasts. Generally the induction of L forms was facilitated by an increase in the osmolarity of the medium (Sharp, 1954; Lederberg & St. Clair, 1958; Landman et al., 1958). The nature of the induction media also influenced the colonial morphology of L forms. In low sodium chloride media Proteus L forms of the colonial type 3A developed (Altenbern, 1961a) but on media containing 2 per cent. sodium chloride, only 3B type colonies developed (Kandler & Kandler, 1956).

Serum.

Mammalian sera have been commonly used to enrich media for the cultivation of bacterial wall defective variants and the Mycoplasma group of organisms. The L_1 organisms of Streptobacillus moniliformis were isolated on rich serum agar media (Klieneberger, 1935). A number of views on the role of serum in media have

been recorded in the literature. Its inclusion in media has facilitated the isolation, growth and propagation of L phases derived from a number of organisms (Dienes, 1948a & b, 1949a, 1967a; Dienes, Weinberger and Madoff, 1950a & b; Dienes & Weinberger, 1951; Poetschke, 1955; Dienes & Sharp, 1956; Minck & Lavillaureix (1956; Altenbern, 1960, 1961a & b; Landman & Ginoza, 1961; Wittler, Malizia, Krammer, Tuckett, Pritchard & Baker, 1960; Kagan et al., 1962; Shchegolev & Prozorovskii, 1963; Hamburger & Carleton, 1966a & b, 1968; Roberts, 1968; Schumann & Taubeneck, 1969; Madoff, 1970). Sera from a number of mammalian sources have been used with varying results. Dienes (1941) obtained L growth of Fusiformis necrophorus on agar containing rabbit serum but similar media exerted an inhibitory effect on streptococcal L forms (Dienes, 1953b). Salmonella L forms grew with difficulty on media containing rabbit serum, but abundant growth was obtained on horse serum agar (Weinberger, Madoff & Dienes, 1950). These investigators also found that L forms adapted to grow on horse serum agar, when transferred to media containing rabbit serum, grew poorly or not at all, but that L strains isolated with rabbit serum grew well on subsequent transfers on rabbit serum agar. Tulasne (1951) noted variations in L growth obtained in media containing bovine, rabbit, human and horse

sera. Wittler et al., (1960) reported that for the isolation of transitional variants media containing 15-20 per cent. human serum was superior to media with horse serum, but for maintaining the viability of these forms, medium containing 20 per cent. rabbit serum was the best of the three. Meningococcal L forms grew equally well on media containing horse, human or rabbit serum (Roberts, 1968). Guinea pig and mouse sera were generally ineffective in aiding the production of L colonies (Weinberger et al. 1950; Dienes & Weinberger, 1951). Bovine serum appears to have fulfilled the serum requirements in the induction media used by Radakova and Androsov (1971) to obtain stable L forms of E.coli. Human and horse sera have generally been found to be the most effective and suitable for use in L induction and propagation media (Weinberger et al., 1950; Nelles, 1955; Minck & Lavillaureix, 1956; Madoff & Dienes, 1958; Hamburger & Carlton, 1966a); horse serum was the better of the two and has been found by some of the above workers to give better yields of L growth. These observations fall in line with the findings of Kalmanson, Hubert, Montgomerie and Guze (1968) who demonstrated that human serum was more rapidly bacteriocidal than horse serum to protoplasts derived from a number of bacterial species. The absolute necessity of serum for the development and propagation

of type 3A L colonies derived from Proteus and Salmonella species has been confirmed by a number of workers (Dienes, 1949a; Weinberger et al., 1950; Altenbern, 1961a). Types 3B L colonies were produced on plates containing penicillin, and serum was not necessary for their induction, but when transplanted on horse serum agar, type 3B L colonies could give rise to type 3A colonies (Weinberger et al. 1950). Serum was essential for the induction and propagation of L forms of Corynebacterium (Poetschke, 1955). Induction media with too high a serum content increased the yield of type 3B L colonies (Altenbern, 1961a). The mode of preparation and treatment of sera have also been reported to affect the production of L growth. Coagulated serum and serum obtained from clotted blood (as compared with defibrinated blood) were unsatisfactory (Dienes, 1949a; Dienes & Weinberger, 1951). Both fresh and inactivated sera have been used with equal success although it had been reported that uninactivated horse serum was inhibitory to L forms derived from Proteus and Salmonella (Nelles, 1955). Weibull and Lundin (1961) however, reported that inactivated sera increased yields of Proteus L forms compared with sera which had not been inactivated. Not all species of bacteria need serum for induction. Proteus mirabilis was grown from a synthetic medium with no added serum (Landman et al., 1958), and in some strains

of Staphylococcus, L forms could be induced in media containing no serum (Kagan et al., 1962; Seligman & Hewitt, 1966); an L form of Bacillus subtilis grew in 7 per cent. sodium chloride broth with no added serum or agar (Burmeister & Hesseltine, 1968). Extremely wide ranges of serum concentrations varying from 1 per cent. to 50 per cent. have been incorporated into L media. Between 10-20 per cent. of horse or human serum has been found to be optimal. While smaller amounts of serum appear to stimulate growth, large serum concentrations were generally found to be inhibitory (Mattman et al. 1960, 1969; Hamburger & Carleton, 1966a).

A number of substances have been tried as substitutes for serum. Edwards (1953) demonstrated that serum could be replaced in the medium for growing the L phase of Proteus vulgaris by a phospholipid faction of egg-yolk and, for growing the L_1 phase of Streptobacillus moniliformis, albumin in addition to the lipid faction was necessary. Bovine albumin has also been substituted for serum with satisfactory results (Friemer et al., 1959; Landman & Halle, 1963; Panos & Hymes, 1964). The L phase of Bacillus subtilis grew well on media in which serum was replaced by 2 per cent. gelatin (Landman & Halle, 1963). Tulasne, Terranova and Lavillaureix (1955) reported that the B group of vitamins and in particular

riboflavine could replace the serum requirements of L phases of bacteria, but Weibull and Lundin (1961) and Lederberg and St. Clair (1958) were, however, unable to confirm the results of the French workers. The failure of L form growth to occur in the presence of serum has been noted in Clostridium tetani and in an unidentified Gram positive spore-bearing bacillus, (Dienes, 1949b, 1950). Mattman, Burgess and Farkas (1958) have made similar observations with a number of Proteus strains in which growth was inhibited by inactivated horse serum. Serum-dependent Gram positive cocci have been adapted over several serial subcultures to grow in the absence of serum (Dienes, 1967a). It has been suggested that the beneficial effect of serum on bacterial L phases was due not to the nutritive value of the serum but rather to its detoxifying properties (Medill & O'Kane, 1954; Mattman et al., 1960; Weibull & Lundin, 1961). The findings of Lorkiewicz (1957, 1958) that carbon could be substituted for serum in growing Proteus L forms would favour this view. On the other hand, Hamburger and Carleton (1966b) have produced evidence that serum, apart from aiding stabilisation of L colonies, also played a nutritive role in promoting higher yields.

Agar.

The characteristic 'fried egg' colonial appearance of Mycoplasma and L growth on solid medium is well known. This expression of L growth is the result of part of its growth developing in the depths of the agar, with a concomitant surface multiplication of the larger colonial components (Dienes & Bullivant, 1967). The small size of the reproductive unit and the lack of a rigid cell wall enable these elements to penetrate the agar actively (Dienes & Bullivant, 1967 ; Dienes, 1968)or passively due to capillary forces, (Razin & Oliver, 1961). Thus the agar penetration by these elements is determined to a large extent by the physical property of the gel. Consequently media solidified with high concentrations of agar, gelatin or silica gel, in which L elements are unable to penetrate, do not allow L colonies to form (Dienes & Bullivant, 1967; Lederberg & St. Clair, 1958). Occasionally with Proteus a few L colonies of type 3B grew on 30 per cent. gelatin by the multiplication of large bodies (Dienes, 1968). The concentration of agar which permits the development of L colonies is largely dependent on the gelling properties of the agar. The use of ordinary grade agar in concentrations of 0.7 to 1.2 per cent. to solidify media has been found to be satisfactory for the isolation and maintenance

of L growth (Hijmans et al., 1969). Better yields of L forms have been reported with more refined agar than with the ordinary granular grade of agar which contained inhibitor substances for L growth (Mattman et al., 1960, 1969). Unidentified inhibitory substances may be present in different lots of the same grades of agar (Nimmo & Blazevic, 1969). A consistant and inverse relationship existed between high agar concentration and L colony yields (Kandler & Kandler, 1956; Landman et al., 1958; Landman & Ginoza, 1961; Weibull & Lundin, 1961; Mattman et al, 1969). Interspecies variations occur in the optimal agar concentrations necessary to achieve the best cell-L transformation. Agar concentrations of between 0.8 to 1.2 per cent. were optimal for Salm.paratyphi (Landman & Ginoza, 1961); in Proteus spp. agar concentrations between 0.7 to 0.8 per cent. appeared to be optimal (Weibull & Lundin, 1961). Agar and osmotic stabilisers have a mutually complementary effect on the production of L colonies of Proteus mirabilis and E.coli, in that at lower agar concentrations, in the absence of a stabiliser, the cell/L form ratio was relatively poorer than it would have been in the presence of an osmotic stabiliser, whereas the unfavourable effects of higher agar concentrations are partly offset by increased osmolarity (Landman et al., 1958). These authors also pointed out the further importance of

agar in L colony development when they demonstrated that the pour plate method gave a better yield of L growth than the streak method. Dienes and Weinberger (1951) stated that type 3A L colonies of Proteus and Salmonella needed soft agar whereas the type 3B L colonies could be propagated in either medium. Altenbern (1961a), however, noted that variations in agar concentrations from 0.6 to 1.2 per cent. were without effect and four separate lots of agar were indistinguishable in their ability to promote type 3A L growth. Increased agar concentrations have been noted to cause reversion of L phases into the bacillary forms. Landman and Halle (1963) found that lysozyme-induced protoplasts of Bacillus subtilis subcultured on 0.7 per cent. agar had, on the average, 31.1 L colonies and no bacillary colonies whereas on 0.9 per cent. agar, the counts were 10.3 reverted bacillary colonies and 22.5 L colonies, and on 2.5 per cent. agar, the corresponding figures were 6.2 bacillary colonies and no L colonies. Similar observations have been made by Hamburger and Carleton (1966b) and Nimmo and Blazevic (1969). Foder and Rogers (1966) promoted reversion of L forms of Bacillus licheniformis by increasing the agar concentrations of the medium.

With few exceptions most L forms derived from bacteria do not grow in liquid media devoid of agar. The L forms of Streptobacillus moniliformis are unique

in this respect in that they are able to grow in a liquid medium without any agar whatsoever (Klieneberger, 1935). Recently a naturally occurring L form of B.subtilis was induced and propagated in sodium chloride broth (Burmeister & Hesseltine, 1968). In some instances transfer of agar blocks bearing L colonies to liquid media has initiated L growth in the latter (Abrams, 1955; Brem and Eveland, 1967; Roberts, 1968). Elimination of agar requirements for L forms and their complete adaptation to grow in liquid media has been described for the L forms of a number of bacterial species (Tulasne, 1950; Dienes, 1970b; Panos & Barkulis, 1959; Altenbern & Landman, 1960; Hamburger & Carleton, 1966a; Winterbauer, Gutman, Turck, Wedgwood & Petersdorf, 1967; Marston, 1968; Stewart & Wright, 1969; Gilpin et al., 1973; Pachas & Currid, 1974; Bertolani et al., 1975).

Divalent cations

The addition of either Mg^{++} or Ca^{++} in appropriate concentrations in induction media has been found to stabilise wall defective variants derived from a number of bacterial species (Weibull, 1956; Lederberg & St.Clair, 1958; Davis et al. 1968; Muschel, 1968). It has been suggested by Davis and co-workers (1968) that these divalent cations prevent the lysis of spheroplasts by forming salt bridges thus protecting

their surfaces from structural alterations. Cheng (1973), however, noted that in Bacteroides ruminicola the cells washed in O.2M Mg^{++} or O.01M Mg^{++}, were totally converted to spheroplasts by subsequent treatment with lysozyme but when such treatment was omitted they remained rod-shaped. However, the cells washed with O.2M Mg^{++} plasmolysed, whereas those washed in O.01M Mg^{++} did not. This would seem to indicate that concentration levels are critical in preventing lysis. Roberts, Ingold, Want and May (1974) found that the omission of Mg^{++} in broth cultures of L forms in no way caused lysis of these forms nor affected their stability. Hamburger and Carleton (1966a) reported that Mg^{++} in L form cultures of staphylococci containing methicillin influenced the subsequent development of L colonies on agar subcultures and accelerated L colony formation. The ions also affected the colony size of these L forms. However, they noted that there was no absolute requirement for this metallic ion. Almost similar findings have been reported by Makemson and Darwish (1972) who observed that though Mg^{++} was not a requirement for E.coli L form induction and growth promotion, Mg^{++} in appropriate concentrations was nevertheless stimulatory. Nimmo and Blazevic (1969) found that Proteus mirabilis L forms after their primary induction could be propagated on media devoid of this metal but this was not so

with a number of Gram positive cocci. Divergent results have been obtained about the ability of Mg^{++} to increase yields of L forms and spheroplasts. Lapinski and Flakas (1969) and Bertolani *et al.* (1975) did not obtain increased yields of L forms or spheroplasts with added Mg^{++} but Altenbern (1961a) found that alteration in magnesium sulphate concentrations had a striking effect on cell to L ratio yields for the type 3A L forms of *Proteus mirabilis*. Diena *et al.* (1964) noted that the addition of traces of Mg^{++} was necessary for the optimal production of glycine-induced spheroplasts of *Salm.typhi*. While Mg ions were not significant in increasing yields of L forms of *Salmonella typhimurium* in growth media of high agar concentrations, the addition of 0.01 per cent. $MgSO_4$ and 10 per cent. sucrose significantly increased L form yields in induction media of low agar concentrations (Shchegolev & Prozorovskii, 1963). The presence of Mg^{++} appears to be critical for the development of pneumococcal L forms (Madoff & Dienes, 1958). The findings of Bertolani and associates (1975) suggest that high magnesium ion concentrations may be useful in preventing reversion of L forms into the bacillary phase. The substitution of Mg^{++} with Mn^{++} has been reported to be equally effective in stabilising and promoting L growth (Landman *et al.* 1958;

Landman & Ginoza, 1961). More recent studies indicate that Ca ions are more effective than Mg ions in the induction and promotion of L growth (Landman & Ginoza, 1961; Makemson & Darwish, 1972) although excess of Ca^{++} in the media is inhibitory (Iannetta & Wedgwood, 1967).

Effects of varying concentrations of inducing agents.

In a number of bacterial species L forms can be produced with inducing agents at concentrations which are normally inhibitory to the parent bacilli. A linear relationship between the concentration of the inducing agent and L form production does not always exist. The effect of high and low concentrations of the inducing agents on the yield and biological properties of the L forms appear to have been not much investigated except in the case of penicillin.

Working with E.coli, Lederberg and St.Clair (1958) obtained higher yields of L colonies with 1,000 units of penicillin/per ml of medium than with 100 units penicillin/per/ml and speculated on the possibility of obtaining still higher yields with 10,000 units penicillin/ml of medium. Medill-Brown and Hutchinson (1957) working with Proteus mirabilis observed that higher yields of L colonies were obtained with increasing amounts of penicillin but at concentrations above 4,000 units penicillin/ml, the

rate of increase of L colony induction declined with a given inoculum size and furthermore there was a concomitant decrease in the size of the L colonies. At around 10,000 units penicillin/ml a definite decrease in L colony production was evident. A similar quantitative relationship between the concentration of the inducing agent and L production yields was apparent in Shigella flexneri (Abbate et al., (1973). Streptococcus faecalis also showed variation in the yield of protoplasts when penicillin concentrations were varied (Kubota, Montgomerie, Potter, Kalmanson & Guze, 1966). Seeberg and Brøson (1974) obtained maximum E.coli L colony growth at the lower concentrations of penicillin and ampicillin.

Watanakunakorn (1971a) used 100 to 1,000 µg/ml concentrations of cycloserine to induce L forms in Staphylococcus aureus. At a concentration of 100 µg/ml of cycloserine, L colonies grew in small numbers, but most of the growth consisted of vegetative colonies, whereas, when the concentration was increased to 200 µg/ml the numbers of L colonies and vegetative colonies were approximately equal. However, at 400 µg/ml, though there was no appreciable increase in L colony yields, the vegetative colonies were virtually eliminated. Further increases in cycloserine concentrations did not increase L colony yields and in fact it had the opposite effect of depressing L form production. A

similar pattern in L,colony production was noticed when lysostaphin was used (Watanakunakorn et al., 1969b). Low concentration of penicillin yielded good L growth only in media of low osmolarity, while in highly stabilised medium higher concentrations of penicillin were required to achieve similar results. That these two variables are contingent upon each other is most significantly seen in Proteus mirabilis, E.coli and Salm.paratyphi (Landman et al., 1958; Landman & Ginoza, 1961).

Hamburger and Carleton (1966a) did not observe any significant increase in the yield of spheroplasts of Staph.aureus in a salt and serum medium with methicillin at concentrations of 20 µg/ml and 100 µg/ml but, when the concentration of methicillin was reduced to 10 µg/ml, smaller yields of spheroplasts were seen. It is difficult to asses from their work the effect of different antibiotic concentrations on L colony yields, as a number of other variable factors also influenced L colony production; the experimental data presented by them is insufficient to arrive at a satisfactory conclusion on this question of yield in relation to the inducing agent. Changes in penicillin concentrations produced marked L colony growth patterns, particularly in Salmonella and Proteus spp. Weinberger and associates (1950) observed that, on soft serum agar plates containing

penicillin concentrations from 5 to 3,200 units/ml type 3A L colonies of Salmonella spp. developed, these being most abundant between concentrations of 200 and 1,600 units/ml. However, type 3B L colonies were not observed in plates with less than 200 units of penicillin/ml and were most numerous at concentrations of 3,200 units/ml. Altenbern (1961a) also noted that high concentrations of penicillin were more likely to lead to the quantitative conversion of Proteus mirabilis to type 3B L colonies. He ascribed this to the effect of high penicillin concentrations being able to neutralise type 3B colony inhibitors in the media. The effect of various concentrations of penicillin on L colonial morphology has been described by Mattman et al., (1969) who reported that in most Salmonella species very high concentrations of penicillin produced the typical 'fried egg' type L colonies, but with lesser concentrations, L colonies without dense centres were produced and when minimal concentrations of penicillin were used only granular L colonies were obtained.

L forms of some organisms can only be induced within a narrow spectrum of the inducing agents. This was most noticeable with the induction of L forms of Pneumococcus which developed in a narrow range of penicillin concentration. Depending on the strain, concentrations between 0.1 and 0.3 units of penicillin/

ml of medium induced L colony formation in Pneumococcus spp. (Madoff & Dienes, 1958). These workers observed that a difference as small as 0.02 units penicillin/ml often influenced L colony production. Similarly, certain inducing agents had only a narrow range of L transforming capacity. Glycine was able to induce Salmonella typhi spheroplasts only if it were incorporated in the medium between 1.5 to 2.0 per cent. (Diena et al., 1964). Vancomycin has a very narrow L inducing range of activity for Staphylococcus aureus and produced L colonies between 5 and 10 µg/ml (Watanakunakorn, 1971b).

In a large number of bacterial species the concentration of the inducing agents necessary for optimal transformation and production showed variation within the species. With penicillin, the concentration appeared to be critical with some strains of Clostridia (Dienes, 1950), but not with other strains (Scheibel & Assandri, 1959). In Listeria monocytogenes 600 units penicillin/ml was found adequate for L induction in all serotypes except a type 3 human isolate which required 1,000 units (Brem & Eveland, 1968a). A number of investigators have shown that E.coli strains required different concentrations of penicillin for production of L forms (Lederberg & St.Clair, 1958; Schumann & Taubeneck, 1969; Seeberg & Brorson, 1974). Penicillin- and methicillin- resistant strains of

Staphylococcus aureus required higher concentrations of these antibiotics to induce L forms (Kagan et al., 1962).

When two transforming agents were used together to bring about L transformation, the amounts necessary to do so were considerably less than when each was used alone. In the production of Bacillus anthracis spheroplasts with lysozyme and glycine, a ten-fold increase in lysozyme was necessary when it was used as the sole inducing agent (Chatterjee & Williams, 1965). In the production of L forms of Haemophilus influenzae, low concentrations of glycine effectively lowered the minimal L inducing concentrations of penicillin (Want & May, 1975).

Growth inhibitory substances and growth promoting factors.

Medill and O'Kane (1954) were the first to report that a number of medium components used for induction and propagation of L forms had an inhibitory effect on these forms. They identified yeast extract, hydrolysed casein, peptones and vitamins as the inhibitors present in natural media. They also demonstrated that L growth was much more luxuriant in defined synthetic and semi-synthetic media than in natural media with or without added serum. Lederberg and St. Clair (1958) confirmed the inhibitory action of yeast extract for both Proteus and E.coli L forms but for the propagation of staphylococcal spheroplasts

a medium containing yeast extract was more favourable than one containing tryptose (Mattman et al., 1961). Madoff (1970) also found that yeast extract enhanced production of L forms in some strains of Streptococcus MG. Likewise casein hydrolysate was found to be a satisfactory medium component for the propagation of L forms of Proteus mirabilis and E.coli (Landman et al. 1958). These workers found that casein hydrolysate was not only a growth-promoting factor but a growth requirement for E.coli. The addition of vitamin-free casamino acids to be basal medium accelerated the formation of L colonies of Proteus spp. (Medill & O'Kane, 1954), but was without effect for L forms of Pneumococcus (Madoff & Dienes, 1958). The effect of the composition of the medium on the yield of type 3A and type 3B L colonies has been thoroughly investigated by Altenbern (1961a). He noted that certain substances in the media inhibited the appearance of type 3B L colonies of Proteus mirabilis. This inhibitory action in the medium was finally traced to the formation of organic acids, particularly formic acid arising from alkaline oxidation of glucose when the latter was steam autoclaved, together with other medium components in the presence of phosphate buffer salts. Other reducing carbohydrates were also capable of reacting similarly. On the other hand, glucose appears to be essential for the production of Salm.typhi

spheroplasts and a minimal concentration of 0.5 per cent. glucose in the propagating medium has been recommended (Diena et al., 1964). van Boven, Kastelein and Hijmans (1967) reported that phosphate buffers in a semi-synthetic medium composed of vitamin-free casamino acids was unsatisfactory for L phase growth and propagation of Group A streptococci. Better results were obtained when the phosphate buffer was excluded from such media. Panos and Barkulis (1959) have also observed the toxic effects of phosphates on Group A streptococci. Mattman and associates (1958, 1960, 1961, 1969) mentioned a number of inhibitory substances present in natural induction media for L phases of different bacterial species including tryptose, granulated agar, other substances present in unpurified agar and inactivated horse serum. The inhibitory action of ascitic fluid and rabbit serum for a streptococcal L phase has been reported by Dienes (1953b). Riboflavin, reported by Tulasne et al. (1955) to have a stimulatory effect on L phases of bacteria, was without an effect on L forms of E.coli and Proteus spp. (Lederberg & St.Clair 1958), and indeed Medill and O'Kane (1954) found that the addition of 50 µg/ml riboflavin to the basal medium gave a poorer L growth of Proteus spp. Meat extract was found to favour the growth of E.coli L forms (Lederberg & St. Clair, 1958) as also was

charcoal for Proteus and pneumococcal L forms (Lorkiewicz, 1957; Madoff & Dienes, 1958). Nicotinamide greatly enhanced the growth of L forms of Mycobacterium spp. (Mattman et al., 1960).

Gaseous environment.

The gaseous requirements for growth which reflect the respiratory physiology of L forms vary considerably. Many L forms of bacteria grow equally well under both aerobic and anaerobic conditions (Landman et al, 1958; Madoff & Dienes, 1958; Landman & Ginoza, 1961; Nimmo and Blazevic, 1969). On the other hand, Seeberg (1973) noted that yields of L forms from E.coli were better under anaerobic incubation but no such differences were noted in subsequent propagation. Anaerobic incubation appears to be essential for the induction of L forms in Salmonella spp. (Dienes, 1948a; Dienes et al., 1950; Weinberger et al., 1950). In contrast to the observations of the above workers, Landman and Ginoza (1961) did not observe any undue effects on the growth of Salm.paratyphi L forms with increased or lowered O_2 tension. A number of investigators have noted an anaerobic environment necessary for the primary induction of L forms of Group A streptococci but this requirement does not seem to be necessary for subsequent propagation (Sharp, 1954; Dienes & Sharp, Freimer et al., 1959; van Boven et al., 1967). The

The gaseous requirements of the L forms derived from anaerobes appear to be of the same order as those of the parent organisms which is particularly obvious in the induction of L forms from Clostridium spp. (Kawatomari, 1958; Mahony, 1973). Similarly in the pathogenic niesseriae optimal yields of L forms were obtained in the presence of increased CO_2 tension. Reduced O_2 and increased CO_2 tensions which favour the growth of bacillary phases of L.moncytogenes were without effect for the production or propagation of their L forms (Brem and Eveland, 1968a). A similar observation was noted by Mattman et al.(1961) for L forms of Staphylococcus aureus. A CO_2 requirement has also been noted for some strains of Streptococcus MG (Madoff, 1970). Opinions seem to vary regarding the gaseous requirements for L forms of Mycobacterium spp. Willet and Thacore (1966) observed that L forms of Mycobacterium tuberculosis developed only in the presence of 10 per cent. CO_2, there being no growth under anaerobic incubation and bacillary growth in ordinary aerobic conditions. Mattman et al. (1960), on the other hand, did not observe any such special requirements for L forms derived from different mycobacterial species (including Mycobacterium tuberculosis) except that anaerobiosis favoured their development. In a recent communication, Seeberg and Brorson (1974) reported the effects of gaseous

environment on L colony morphology. An E.coli strain produced the typical 'fried egg' type of L colonies under aerobic incubation but in a CO_2-supplemented environment a homogeneous L type growth resulted. These workers also noted that there were less bacillary colonies on anaerobic induction media than in aerobic plates and subsequently more L colonies were produced in the plates incubated anaerobically. Aerobic rather than anaerobic incubation favoured the development of type 3A L colonies in Proteus (Dienes, 1949a).

Effects of temperature of incubation.

The most widely used temperature range for the induction of L forms from a large number of bacteria appears to be 34-37°C. Nimmo and Blazevic (1969) recommended a temperature of 35°C for the induction of L phases. In Proteus and Salmonella spp. a broth culture after 3 hours' incubation at 37°C has been reported ideal for L transformation (Klieneberger-Nobel, 1960). L forms of E.coli have also been induced at 37°C (Lederberg & St.Clair, 1958; Seeberg & Brorson, 1974). Roberts (1968) reported maximum yields of gonococcal L forms when parent cultures were incubated between 35-36°C and for meningococcal L forms between 34-38°C. Madoff (1970) obtained satisfactory L growth of Streptococcus MG from 34°C incubated cultures. Pachas and Currid (1974) induced

L transformation in Erysipelothrix rhusiopathiae cultures incubated at 33°C. Ørskov (1942) chose a temperature of 32°C in his study of the development of L colonies from Streptobacillus moniliformis as growth at this temperature was slower than at 37°C. In an experiment using temperature gradient plates it was found that L forms of Salm.paratyphi gave a good growth throughout a temperature range of 31-40°C, (Landman & Ginoza, 1961). However, these investigators preferred an incubation temperature of 30° rather than 37°C as L growth at the latter temperature was inconsistent. Pseudomonas aeruginosa L forms were induced both at 25°C and 37°C, but subsequent refrigeration enhanced L colony formation particularly if returned to 25°C incubation (Edman & Mattman, 1961).

In Listeria monocytogenes L form induction was most favourable at room temperature although incubation at 37°C gave comparable results, but at 4°C L colony formation was retarded at the granular stage of development (Brem & Eveland, 1967, 1968a). Edman, Pollock and Hall (1968), however, noted that L forms of Listeria monocytogenes grew over a wide temperature range between 10-40°C but the optimal induction was at 30°C. Altenbern and Landman (1960) noted the effects of incubation temperature on colonial morphology of L forms of Proteus mirabilis and found that type 3A L colonies of this organism were greatly favoured at

25°C and 30°C. Subsequently Altenbern (1961a & b, 1964) found that transformation of type 3A to type 3B L colonies of Proteus mirabilis began earlier and proceeded more rapidly at 37°C than at 30°C. Furthermore, it was also shown by him that the stability of the type 3A L forms was greatly affected by temperature of incubation, a reversion to the bacillary state being fostered more readily at 37°C than at 30°C.

Effects of hydrogen ion concentration.

There is very little information on the effects of hydrogen ion concentration on the induction and propagation of L forms. In the studies of Nimmo and Blazevic (1969), the medium that proved to be the most suitable for the induction of L forms from 13 strains of bacteria representing 6 genera was the medium which had a final pH of 7.6 to 7.8. For the isolation of L forms and wall defective variants from clinical specimens a medium with pH adjusted to 7.8 has been found satisfactory (Gutman, Turck, Petersdorf & Wedgwood, 1965). Minck and Lavillaureix (1956) recommended an induction medium with an alkaline reaction nearing pH 8. Klieneberger (1936) noted that the L1 organisms preferred a more alkaline reaction of 7.6-8.0 for their development. Watanakunakorn (1971a) used a medium with pH 7.0 for the induction of staphylococcal L forms while,

for the production of spheroplasts of Salm.typhi, a liquid medium of pH 7.2 proved adequate (Diena, Wallace & Greenberg, 1965). For the induction of Proteus mirabilis the medium used by Teuber (1969) had a pH between 7.3 and 7.5 but for E.coli an induction medium of pH 6.7 has been used (Makemson & Darwish, 1972). L forms of Listeria monocytogenes have been isolated on a medium of pH 7.1 to 7.3 (Brem & Eveland, 1968a). Lederberg and St. Clair (1958) found that the optimum pH requirement for E.coli L forms was 6.3. Landman and co-workers (1958) found that, in the case of Proteus mirabilis, pH values between 5.5.and 8.0 were without effect on L colony formation or counts except for a higher cell L ratio at the top and bottom end of the range, but, for the development of E.coli L forms, a much narrower range of 6.1 to 7.2 was more suitable; no growth of L forms of E.coli occurred at pH 7.5 though at pH 6.0 and below the cell L ratio was greater. These workers also noted that low agar concentrations coupled with low pH values supported far less L growth than low agar concentrations at a neutral pH. Seeberg and Brorson (1974) observed a quantitative relationship between L form production and pH for different strains of E.coli; while some strains gave optimal yield of L growth at pH ranges of 5.0-6.0, other strains did so in the range pH 6.0-7.0. In Mycobacterium spp.

L forms the hydrogen ion concentration was particularly important when impurities were present in the agar, or serum of human origin was used in the medium, or when salt was substituted for sucrose to provide the required osmolarity (Mattman et al., 1960).

van Boven and associates (1967) noted considerable loss of viability of the L elements derived from Group A streptococci when the pH of the medium dropped to 5.0 or 5.2 but these forms could be gradually adapted to grow in these unfavourable pH conditions.

Strain differences.

Differences exist between strains in their ability to produce L phase growth. Medill-Brown and Hutchinson (1958) found that L transformation in Proteus mirabilis took place only above concentrations of 40 units penicillin/ml in the medium whereas Teuber (1969) was able to induce L growth in this species with 2.5 units of penicillin/ml. The unusually low concentration necessary to induce L transformation in this species appears to be a property of the strain. Ward and Martin (1962) studied the ability of the two different antibiotics to induce L phase growth in a number of Gram positive and Gram negative organisms. Using the antibiotics separately, they found that, of the 25 Group A streptococci tested for the ability to induce L transformation, only 2 strains did so with both the antibiotics; in Staphylococcus spp. only 4 of the

50 strains used responded to L induction with either of the antibiotics and the figures for Salmonella spp. were 3 of 9 strains. Smith and Willis (1967) reported that, of the 20 penicillin-sensitive strains of Staphylococcus aureus exposed to penicillin, in only 6 could L colonies be induced. Kagan, Prozorovskii and Koptelova (1963) made similar observations with Gonococcus, haemolytic streptococci and Salmonella spp.

Penicillin- and methicillin-resistant strains required higher concentrations of these inducing agents for L transformation than the penicillin-sensitive strains (Kagan et al., 1962).

Dienes and Sharp (1956) noted that even in the presence of high concentrations of the inducing agent the osmotic requirements for L induction in different strains varied. While some strains could be induced into L phases with 0.26M sodium chloride concentrations, the vast majority of the other strains required an osmolarity of 0.43 to 0.51M sodium chloride. They also observed that these strains varied when the sodium chloride was replaced by other salts. One strain produced L colonies when the required tonicity was provided either by disodium hydrogen phosphate or calcium chloride, but a second strain produced L colonies on a medium containing high calcium chloride concentrations but not on one containing disodium

hydrogen phosphate, while the third strain grew on a medium with high disodium hydrogen phosphate content but not one with high calcium chloride content. Freimer et al. (1959) noted that more Group A streptococcus strains could be induced into L forms if the parent strains were first adapted to grow in an hypertonic environment before induction was attempted. In E.coli, while some strains yielded L colonies, others were unproductive (Dienes & Sharp, 1956; Lederberg & St. Clair, 1958; Artemieva, 1967; Seeberg, 1973; Seeberg & Brorson, 1974). While many strains of E.coli transformed into protoplasts, not all the cells were capable of transforming into L colonies (Lederberg & St.Clair, 1958); Seeberg (1973) successfully induced L growth in 47 strains of E.coli but of these only 9 could be satisfactorily subcultured, while in the vast majority no more than a single subculture was possible. Madoff and Dienes (1958) made similar observations with pneumococci. Landman et al. (1958) found that in 4 different strains of Proteus mirabilis requirements for optimal osmolarity varied with the strains and they all gave good yields at their respective optimal environmental conditions. Parallel observations have been made on the optimal hydrogen ion concentrations that are conducive to maximal L colony yields in the different strains of E.coli (Seeberg & Brorson, 1974). It has been

suggested that the strain differences observed in connection with the difficulty of inducing L forms may be related, in parts, to the antigenic structure of the organism (Edman et al, 1968).

Miscellaneous factors.

(i) Age of inoculum. Opinions differ as to whether the physiological state of the organisms at the time they are exposed to the action of the inducing agents affect L transformation. Kandler and Kandler (1956) found that the age of the cultures used as inoculum for L colony transformation significantly affected cell/L ratios. Medill-Brown and Hutchinson (1957) reported that the ability of Proteus mirabilis to form L colonies in the presence of a constant amount of penicillin decreased slightly at the lag phase of growth. On the other hand, Landman et al. (1958) did not observe any significant changes in cell/L ratio using 24, 48 or 72 hour cultures. No striking differences were noted in the total yield of Salm. paratyphi L colonies using cells either in the stationary or the lag phase. Minck and Lavillaureix (1956) claimed to have obtained good results with different bacteria using cells that had completed the exponential phase of multiplication. The age of the culture was not a critical factor in the induction of L forms from Mycobacterium spp. (Mattman et al., 1960).

(ii) Methods of culturing. Landman and co-workers (1958), using Proteus mirabilis, studied L colony yields obtained by pour plate and streak plate methods. In the synthetic medium they used the cell/L ratio for the pour plate method was between 3.3 and 5.8 while in the corresponding streak plates no L colonies were produced even at very much lower dilutions. In the complex medium, the cell/L ratio for the streak plate was 3, while the corresponding figure for the pour plate method was 1.0. Weibull and Lundin (1961) also found L colony counts of Proteus mirabilis much lower in the streak plates than in the pour plates, even though the samples were identical. In Listeria monocytogenes more colonies were obtained in pour plates than on streak plates (Brem & Eveland, 1968a). Lederberg and St.Clair (1958) were unable to get surface growth of E.coli L forms although in pour plates they grew abundantly. Dienes and Weinberger (1951) also noted similar requirements for the isolation and propagation of type 3B L colonies of Proteus and Salmonella spp., but in the case of pneumococci the requirements were just the reverse in that induction of pneumococcal L forms occurred hardly at all in pour plates and their propagation in pour plates was limited to one or two passages (Madoff & Dienes, 1958). The use of a bottom layer underneath the inoculated layer was found to be essential in obtaining good yields of L

colonies from haemolytic streptococci (Gooder & Maxted, 1961). A *Proteus* strain yielded more L colonies on the surface than between agar layers (Bonifas, 1954). Good growth of L colonies from haemolytic streptococci was obtained when a piece of sterile filter paper was placed on the surface of the medium during incubation (Gooder & Maxted, 1961) but, for the induction of L forms from staphylococci, this was without any effect (Williams, 1963).

(iii) *Inoculum size*. A heavy inoculum was found necessary to induce the L phase from the bacillary forms of *Listeria monocytogenes* (Brem & Eveland, 1968a). Weibull and Lundin (1961) also noted similar requirements for induction of L forms of *Proteus mirabilis* in serum-free medium.

(iv) *Shaking of cultures*. Better yields of spheroplasts and L forms have been observed in liquid cultures which have been agitated during incubation (Dienes *et al.*, 1964; Brem & Eveland, 1968a).

Quantitative yields.

Gooder and Maxted (1961) pointed out that, under the best of conditions, transformation of a coccus into its L form by the combined action of penicillin and an amino acid such as glycine would not be more than

0.00001 per cent., whereas a muralytic enzyme attacking the cell wall would, under similar circumstances, give between 1 to 100 per cent. yield of L colonies. Watanakunakorn *et al*. (1969b), however, could only obtain a maximum L colony yield of 1.0 per cent. using the muralytic enzyme lysostaphin on *Staphylococcus aureus*. In the Gram negative species, although a similar comparison is not applicable, it has nevertheless been shown that 100 per cent. conversion of the bacillary forms of *Proteus* and *Salmonella* into their L phases could be achieved by the action of cell-wall inhibiting antibiotics (Landman *et al*., 1958).

Table I gives the quantitative yields of L colonies from various bacterial species obtained by different workers.

TABLE I

Species	Yield of L colonies. % of bacteria transformed into L phase	References
Proteus spp.	0.001	Kandler & Kandler (1956)
	1.0	Taubeneck (1962)
	10.0	Liebermeister & Kellenberger (1956)
	10.0-74.0	Bonifas (1954)
	100.0	Landman et al. (1958)
E.coli	50.0	Lederberg & St.Clair (1958)
	72.0	Makemson & Darwish (1972)
	100.0	Landman et al. (1958)
Salm.paratyphi	5.0-50.0	Landman & Ginoza (1961)
Shigella flexneri	2.6	Abbate et al. (1973)
Bacillus subtilis	100.0	Landman & Halle (1963)
	100.0	Landman, Ryter & Knott (1964)
Clostridium tetani	0.0005	Scheibel & Assandri (1959)
Staphylococcus spp.	0.002-0.7	Hamburger & Carleton (1966a)
	1.0	Watanakunakorn et al. (1969b)
	100.0	Seligman & Hewitt (1966)
Group A Streptococcus	12.0	Freimer et al. (1959)
	1.0-100.0	Gooder & Maxted (1961)
Group D Streptococcus	0.01	Kubata et al. (1966)
	10.0-100.0	King & Gooder (1970a)
Neisseria meningitidis	1.0-10.0	Bohnhoff & Page (1968)
Pseudomonas aeruginosa	0.0005-3.0	Bertolani et al. (1975)

REPRODUCTION

The mode of reproduction in the L phases of bacteria is still a highly controversial field and a number of investigators have expressed divergant views on this cytological aspect. The basic difference appears to be centred on the genesis and multiplication of the large bodies and the smaller forms, the latter comprising granules and elementary corpuscles. The first accounts of the reproductive physiology of L forms was by Klieneberger (1935, 1936). She considered that the development of L_1 colonies began with granules growing into filaments and the latter forming a reticulum. Pear- and bulbous-shaped forms developed on this network and with further incubation these round bodies divided and produced granules, the process being essentially the same in liquid media as on solid media. When she found subsequently that the fine filaments making up the network were not genuine structures (Klieneberger, 1940)she proposed an alternative explanation of the reproductive processes (Klieneberger-Nobel, 1949a, 1951). The basic reproductive elements were still the granules (elementary corpuscles) which originated from the bacilli. These corpuscles consisted of intensely staining chromatinic materials surrounded by a narrow rim of protoplasm. In the process of development the chromatinic centre formed a network which ultimately segregated into a number of smaller units, thus giving

rise to a multinucleate body from which, by a process of segmentation of the protoplasm, several separate units were formed each with its own chromatinic centre. According to Klieneberger, the surrounding cytoplasm usually faded away resulting in "filterable and minimal reproductive units". She had always maintained that these units were viable and were the starting point of L growth. As in her earlier observations she reported that in liquid media the process of development was not unlike that described on solid media. Her explanation of the presence of large bodies was that these arose as a result of fusion of the granules forming disc-like bodies equivalent to what were called large bodies by her contemporary workers. Heilman (1941a) followed the development of the L_1 organisms of Streptobacillus moniliformis in slide cultures. He, too, noted that the granules were capable of growth and reproduction. He claims to have observed the enlargement of these elementary granules into larger structures which were refractile and disc-like. This disc-like structure enlarged and became less opaque revealing large numbers of granules. The spherical structures ultimately burst as a result of the tremendous increase of granules, thus setting free the tiny elements. This process of development was common to both liquid cultures and surface cultures on solid media. An altogether different view

on reproduction of L forms was put forward by a number of other workers who observed that bacilli under the influence of L inducing agents or undergoing spontaneous transformation always swelled up to produce large bodies (Dienes, 1939a, b, c, 1942, 1943, 1946a, 1949a, 1960, 1967a, 1968, 1970c; Dienes & Weinberger, 1951; Dienes & Madoff, 1966; Dienes & Bullivant, 1967). These large bodies possessed three potential courses of development, namely they could replicate themselves as large bodies, or reproduce small granules or transform back into the bacillary phase from which they were originally derived. The reproduction of large bodies as large bodies has been noted in liquid media (Dienes, 1967a; Dienes & Bullivant, 1967) and on solid media as in the case of Serratia (Bandur & Dienes, 1963) or when grown on gelatin, or membrane filters (Dienes, 1967a; Dienes & Madoff, 1966); on solid media such as gelatin or silica gel they increased in size and produced irregular outgrowths which eventually became detached. When grown on the top of membrane filters they penetrated the pores of the membrane and branched out in a filamentous manner but on contact with the agar they reproduced as granules, which penetrated into the agar. The reproduction of granules from large bodies occurred particularly on soft agar. The large body enlarged

and from one or several points on its surface, granules grew out and penetrated the medium. The granules appeared as rounded, elongated or bipolar forms or even as tiny curved filaments (Dienes, 1949a; Dienes & Weinberger, 1951). The third potential course of development of large bodies was the production of bacilli which was reported by Dienes in some of his early publications (1939c, 1943). Bacteria were not only produced within the large bodies (Dienes, 1939c, 1943, 1946a; Dienes & Weinberger, 1951) but arose either by elongation of the large body into filaments which segmented into several bacillary forms or by segmentation and irregular division of the large body into several fragments which, with further development and reproduction, emerged as bacilli of normal morphology (Minck & Lavillaureix, 1956; Dienes, 1970c). The granules in L colonies have been regarded by some as fundamental reproductive units in the cyclical development of L phases of bacteria (Klieneberger, 1935, 1936; Klieneberger-Nobel, 1949a, 1951; Tulasne, 1951). That the granules were able to replicate themselves directly without necessarily transforming into another morphological form, has been observed by Minck and Lavillaureix (1956), Dienes (1967a, 1970c) and Dienes and Bullivant (1967). Weibull (1963) has also reported the multiplication of granules

on agar but this has been observed only in granules larger than 0.6μm. Granules could transform into larger elements either by increase in size (Dienes, 1939a, 1968, 1970c; Dienes & Weinberger, 1951; Tulasne, 1951) or by fusion of one or more of these granules (Klieneberger-Nobel, 1949a). Electron microscopic studies have revealed another morphological element present in L cultures whose origin and reproductive capabilities are not fully known. These bodies have been called 'elementary corpuscles' measuring from 0.05 μ to 0.1 μ and do not appear to contain DNA (Dienes & Bullivant, 1967, Dienes, 1970c). It has been suggested by these workers that they either originate from within the large bodies or even from growth of the granules, but conclusive evidence of their origin is still lacking. Hijmans et al. (1969) tend to view the granules as non-functional debris incapable of any sort of reproduction. Weibull and Lundin (1962) observed that L elements with a diameter measuring between 0.6 and 0.7 μm were capable of forming micro-colonies. Weibull and Beckman (1961) were of the opinion that the small bodies having a diameter less than 0.3 μm found in stable L Proteus cultures were unlikely to be able to form colonies or multiply as these elements had a low biosynthetic activity and lacked DNA. Small bodies of streptococcal-L forms also appear to lack DNA and ribosomes according to

Coussons and Cole (1968) who doubted the ability of these forms to multiply. Little comment has been made on the role of intracellular granules found within the large bodies. Dienes (1939a) did not see any relationship between these granules and L colony formation, but in recent studies (Dienes, 1967a) it has been shown that the granules were viable bodies capable of multiplication in suitable media. By using micromanipulation techniques Roux (1960) found that the granular elements released from L bodies never gave rise to L colonies. Cytochemical studies have shown that these intracellular granules contain DNA and hence their reproductive capabilities cannot be ruled out completely (Li Hui, Li Tien-Lin & Lei Ai-Te, 1964).

In liquid cultures the granules are incapable of multiplication in most instances (Dienes & Bullivant, 1967) but in cultures which have undergone permanent alterations, multiplication of small granules in broth has been recorded (Dienes, 1970b).

A completely different view in the formation of large bodies has been put forward by Stempen and Hutchinson (1951) who reported that the formation of large bodies was either a process of budding of the bacilli which increased in size and absorbed the contents of the rod, or, in a number of instances, by the fusion of two such forms. The resultant

large bodies regenerated bacilli or less often multiplied to form colonies. These workers also observed the presence of intracellular granules in the large bodies.

Liebermeister (1960) was of the opinion that the large bodies were formed, not by the fusion of rods (Stempen & Hutchinson, 1951) or of granules (Klieneberger-Nobel, 1949a), but by the fracture of the bacterial cell surface and extrusion of its contents through this cleavage (Liebermeister, 1954; Liebermeister & Kellenberger, 1956). His time-lapse microphotographs of such a development do not appear to be very convincing but they illustrate well the process of sprouting of the large bodies. Freundt (1950) observed that, under the influence of high concentrations of penicillin, Proteus rods produced buds which increased in size to become spherical and later developed pseudopodium-like outgrowths. These eventually burst, liberating granules which appeared to be incapable of further development. However, he claimed that the globoid structures produced in the early stages of rod transformation were capable of multiplication and he equated these structures with the large bodies described by Dienes (1946b). The chief elements of the L colonies according to Freundt were 'polymorphous highly refractive yellowish brown elements'. From his

description of the development of L colonies it appears that he was uncertain how these forms were produced. He reported that they were found in close association with the large spherical forms and he suggested 2 modes of development for the polymorphous elements, (a) by the transformation of the newly developing spherical bodies and (b) by the constriction and division of the more mature spherical elements, although he considered that these changes never occurred in spherical forms which have become very large or have developed vacuolation. The 'polymorphous elements' divided by transverse fission, growing down into the agar. These elements, if transferred to a medium containing no inducing agent, readily produced the bacillary forms.

Tulasne (1951) proposed a cyclical development for the different morphological elements found in L colonies. He considered the fundamental unit of reproduction in L colonies to be the 'dwarf' forms which were capable of increasing in size to form globular (large) bodies that in turn later divided to form the dwarf forms. His observations on the cyclomorphic development of L colonies share many similarities with the findings of Klieneberger-Nobel (1949a, 1951). Tulasne (1953) also described four methods of reproduction of bacilli from the L elements in Proteus, (a) the L form ruptured,

liberating rods, (b) the L form divided longitudinally into 5 or 6 filaments, (c) the L form grew into a fusiform cell from which rods eventually broke off and (d) the L forms elongated, branched and broke into small rods. Kellenberger, Liebermeister and Bonifas (1956) were not convinced of the ability of dwarf forms to replicate. According to them these were protoplasmic fragments devoid of any reproductive capabilities. Taubeneck and Gumpert (1967) claimed that there were differences in the development of L forms from rod shaped bacilli. In the development of unstable L forms on media containing penicillin, rods transformed into spheres which multiplied in the agar, whereas in stable L form production, the cell envelope allowed the escape of cytoplasm which, if it succeeded in gaining entrance into the agar, reproduced by budding smaller elements. It is quite possible that what these two workers suggested was not merely as escape of the cytoplasm but probably an extrusion of the cytoplasmic contents together with its limiting membrane through a cleavage in the outer wall, because it is inconceivable that cytoplasm <u>per se</u> is capable of penetration nor, for that matter, of performing any physiological function.

FILTERABILITY

One of the fundamental characteristics which differentiate bacteria from mycoplasmata and viruses is the ability of the latter groups of organisms to pass through filters that hold back bacteria.

That the L phases of bacteria were filterable was first demonstrated in Streptobacillus moniliformis L_1 organisms (Klieneberger, 1936). Two methods have generally been used to demonstrate the ability of L organisms to pass through filters. The first method (ordinary filtration under positive or negative pressure) has been mainly used not only to demonstrate the presence of viable filterable elements but also to measure their size and numbers present in the filtrate. In the second method the ability of the L forms to grow through filter membranes of different porosities placed on a suitable medium is assessed. Most of the early investigations on the filterability of L phases of bacteria have been carried out with filter candles.

While Klieneberger (1936) was able to obtain easy filtration of the L_1 organisms through Berkefeld V filters, Heilman (1941a) obtained inconclusive results. However, the filterability of L phases of bacteria has been confirmed by a number of other investigators (Silberstein, 1953; Carrère, Roux & Mandin, 1954; Kellenberger et al., 1956; Tulasne & Lavillaureix,

1958; Rada, 1959; Panos, Barkulis & Hayashi, 1960; Williams, 1963; Mortimer, 1965; Molander, Weinberger & Kagan, 1965; Dienes & Madoff, 1966; Coussons & Cole, 1968; van Boven Ensering & Hijmans, 1968; Roberts, 1968).

In Streptobacillus moniliformis L forms, the smallest viable elements obtained by filtration through gradocol membranes were estimated to be between 0.175 and 0.35μm (Klieneberger-Nobel, 1949b). Mortimer (1965) found that viable L elements of Group A Streptoboccus passed through Millipore filter membranes with an average pore diameter (A.P.D.) 0.45μm, while Panos and associates (1960) obtained viable units measuring 0.3μm. van Boven et al. (1968) obtained viable L form elements of Group A streptococci measuring 0.45μm to 0.65μm. Roberts (1968) noted that meningococcal L forms will pass through Millipore filter of 0.45μm and 0.30μm A.P.D. but were retained completely by membranes of 0.22μm and 0.10μm A.P.D. Williams (1963) using gradocol filters of different porosities found that viable L elements of Staphylococcus aureus were unable to pass through filters of less than 0.7μm A.P.D., whereas Dienes and Madoff (1966) were able to demonstrate the ability of staphylococcal elements to penetrate through 0.1μm A.P.D. filters placed on agar. The ability to grow through filters of different porosities has been investigated for a number of L forms derived

from other bacterial species. The sizes of the smallest viable L unit determined by this technique for Proteus L forms were 0.1 to 0.2μm (Tulasne & Lavillaureix, 1958), >.22μm (Dienes & Madoff, 1966) and 0.5μm to 0.75μm (Silberstein, 1953). For Staphylococcus L forms the corresponding measurements were 0.1μm (Dienes & Madoff, 1966), 0.05μm to 0.45μm (Molander, Weinberger & Kagan, 1965) and 0.1μm to 0.2μm (Smith & Willis, 1967).

The accuracy of measurement by filtration techniques is subject to gross errors due to the plasticity of the L elements; furthermore, low filtration recoveries necessitate the use of inocula with high titres to avoid negative results. The low recovery of L elements following filtration has been ascribed to adsorption of these elements to the filters (Hijmans et al., 1969). Improved filtration recoveries have been achieved by sonic treatment prior to filtration (Panos et al., 1960) or pre-treatment with deoxyribonuclease (Coussons & Cole, 1968). Weibull and Lundin (1962) have drawn attention to a number of factors which seriously limit the usefulness of filtration techniques in determining the size of the smallest viable elements in the L phases of bacteria.

REVERSION

It was the ability of L_1 organisms of Streptobacillus moniliformis to revert to the bacillary phase that led Dienes (1939a) to consider that the L_1 organism was a growth variant and not an unrelated symbiont as Klieneberger (1935) had suggested. He repeatedly observed not only the development of these mycoplasma-like elements developing from bacteria, but also their reversion to the bacillary phase. There is general agreement that the large bodies found in L colonies produce the bacillary elements under appropriate cultural conditions (Dienes, 1939c, 1943, 1946a, 1960, 1968, 1970c; Dienes & Smith, 1944; Dienes & Weinberger, 1951; Stempen & Hutchinson, 1951; Tulasne, 1953; Medill & Hutchinson, 1954; Minck & Lavillaureix, 1956).

Reversion, therefore, is a process whereby the large bodies, instead of producing L elements, follow the alternative course of development in reproducing the bacillary forms. L forms that easily revert to the bacillary phase are known as unstable L forms but those that continue to propagate as L forms in the absence of inducing agent are known as stable L forms. In bacterial species that produce types 3A and 3B L colonies the latter often reverted more readily than the type 3A L colonies (Dienes, 1949a; Dienes & Weinberger, 1951;

Minck & Lavillaureix, 1956). The terms "stable" and "unstable" forms are not interchangeable, however, with type 3A or type 3B L colonies. The process of reversion and the factors that stimulate reversion are discussed below.

Transfer to liquid media.

The L_1 organisms of Streptobacillus monilformis, when grown in liquid media, transformed into the bacillary phase (Dienes, 1939a, 1942). Dawson and Hobby (1939a) and Brown and Nunemaker (1942) have also observed that L_1 growth, when transferred to liquid media, invariably resulted in reversion. Clostridium perfringens L colonies when propagated in liquid media reverted to the original state within 24 to 72 hours (Kawatomari, 1958). Nimmo and Blazevic (1969) have also noted that reversion occurred more readily in liquid media. Established L forms of Group A streptococci that had been subcultured several times on penicillin medium, when propagated in penicillin-free liquid medium, produced bacilli, the process being hastened by the addition of penicillinase to the liquid medium. Mattman et al. (1961) however, found that reversion was more readily achieved on solid media than in liquid media for streptococcal L forms.

Withdrawal of the transforming agent.

Reversion from L phases to the bacillary phase has been shown to occur with nearly all species when the transforming agents were withdrawn, particularly in the unstable L forms or in the newly isolated L forms. In bacteria that produce types 3A and 3B L colonies, the withdrawal of the inducing agent resulted in the rapid reversion of the latter colony types to the bacillary state within a few hours (Dienes, 1949a; Dienes & Weinberger, 1951). Although the type 3A L colonies do propagate on penicillin-free media, bacilli reappear in them if they are allowed to age or kept for long periods without transferring them on to fresh media.

Silberstein (1953) pointed out that in L forms that reverted the concentration of the antibiotic used for induction had a marked influence on the capacity to revert. Thus Proteus L forms on agar containing 1,000 units penicillin/ml could be transferred at weekly intervals without reversion occurring, while on media containing 10 units penicillin/ml swarming of bacilli on the plates occurred in 2-3 days, thus necessitating daily transfers. Seeberg (1973) working with L forms of E.coli noted that even with high concentrations of penicillin, the propagation of L forms in all strains was not easily achieved. In Salmonella paratyphi on the other hand L phase induced by high concentrations showed almost no reversion

(Landman & Burchard, 1962). The sporadic appearance of revertants in the presence of high concentrations of antibiotics has been noted in streptococcal L forms induced in this way (Dienes, 1953b).

Occasionally L forms induced on antibiotics are able to undergo serial propagation as altered variants in antibiotic-free medium only in a limited number of transfers. Kagan et al. (1962) observed that revertant staphylococci reappeared in methicillin-induced L forms after 2-3 passages on methicillin-free maintenance medium. A similar observation was recorded by Williams (1963) who found revertants reappearing in a strain of penicillin-induced staphylococcal L forms that had been passaged several times in penicillin-free medium. Schönfeld (1961) doubted the existence of stable L forms in Staphylococcus spp. as even 200 passages of the L forms derived from this species reverted on penicillin-free medium. The mere exclusion of penicillin or other inducing agents does not always bring about reversion in the more established L forms. This phenomenon has been reported for a number of bacterial species by different workers.

Gelling agents.

It was mentioned earlier in this review that a gelling agent such as agar was necessary for the

characteristic expression of L growth. However, increasing the hardness of the solid medium either by incorporating large amounts of gelatin or by increasing the concentration of agar has been shown to favour reversion. Non-quantitative reversion of L forms has been noted by a number of workers when the concentration of agar in the inducing or propagating medium was increased (Hamburger & Carleton, 1966b; Foder & Rogers, 1966; Roberts & Wittler, 1966; Gooder, 1968b; Nimmo & Blazevic, 1969). Landman and Halle (1963) reported the quantitative mass reversion of Bacillus subtilis protoplasts and L forms when agar (2.0 to 2.5 per cent.) or gelatin (15 to 35 per cent.) were employed as the solidifying agents. On L form media solidified with 15-25 per cent. gelatin, spheroplasts and protoplasts of Group D streptococci reverted quantitatively to the streptococcal vegetative form but no such reversion occurred when stable L forms of the Group D streptococci were treated in a similar manner (Gooder, 1968b; King & Gooder, 1970b).

Serum

The incorporation of serum in high concentrations is known to be inhibitory for L production (Mattman et al., 1960, 1969; Hamburger & Carleton, 1966a). Brown and Nunemaker (1942) noted that L_1 organisms derived

from Streptobacillus moniliformis reverted to the parental form when horse serum in the medium was replaced with ascitic fluid. Dienes (1953b) also reported that in media in which the inducing agent and horse serum were omitted, streptococcal L forms reverted; it would appear either or both the missing additives were responsible for the reversion in this case. In Salmonella spp. the stability of L forms depended upon the type of serum that was incorporated into the culture medium.(Mattman et al., 1969). These workers observed that when serum from guinea-pigs was used, L colonies reverted rapidly at incubation temperatures from 4 to 37°C, while in media containing horse serum the L colonies remained stable at these temperatures for one month. In Proteus mirabilis decreased reversion of the L forms was observed when the serum concentration in the medium was reduced (Altenbern, 1961b). Hamburger and Carleton (1966b) considered serum to be a more important factor than agar concentration in preventing reversion of staphylococcal L forms. More than 90 per cent. of the staphylococcal spheroplasts in media containing serum formed L colonies regardless of the agar concentrations used, whereas in the absence of serum the agar concentrations strongly influenced the type of colonies that developed; thus, on hard agar, 95 per cent. of the plated spheroplasts reverted to

vegetative forms. However, the stable L forms of Staphylococcus failed to revert irrespective of the hardness of the agar and of the presence of absence of serum.

Effects of the nature and concentration of osmostabilisers.

Gooder (1968b) reported that, on media in which the osmolarity was provided by sucrose, revertant streptococci appeared in the L colonies, but in media where this was substituted with appropriate concentrations of ammonium chloride, no reversion occurred. Nimmo and Blazevic (1969), on the other hand, obtained almost totally conflicting results. They not only found sucrose to be superior to the salts as an osmostabiliser but also that reversion was promoted by a decrease in sucrose concentration in the propagation medium. The substitution of osmotic stabilisers was without effect on the reversion potentiality of Pseudomonas aeruginosa L forms (Bertolani et al., 1975). Lowering the concentration of sodium chloride was reported (Marston, 1961) to aid reversion of staphylococcal L forms, but Hamburger and Carleton (1966b) did not find evidence to support this view.

Miscellaneous factors

Certain ingredients in L media have often been

blamed as agents causing reversion and one of these is yeast extract. Group A streptococci L forms which did not revert when the inducing antibiotic was withdrawn did so when yeast extract was added to the medium (Crawford et al., 1958). A similar observation was made by Marston (1961) with staphylococcal L forms which failed to show reversion when penicillin was omitted and the agar concentration increased, but they readily reverted in the presence of yeast extract. Hamburger and Carleton (1966b) and Williams (1963) were unable to confirm the findings of Marston (1961). Altenbern (1961b) also reported that some constituents of media such as peptone and yeast extract could be varied from 0.1 to 2.0 per cent. without altering the frequency of reversion.

Media containing blood have been reported to cause reversion (Mattman et al., 1961). A comparison made of reversion of Proteus mirabilis L forms in nitrogen-free and nitrogen-rich fresh media showed that reversion occurred whether the medium contained nitrogen or not thus indicating that the endogenous nitrogen provided the necessary substrate for cell wall re-synthesis (Altenbern, 1963).

Ageing of cultures, particularly on media in which the inducing agent had been excluded, promoted reversion (Dienes, 1949a; Dienes & Weinberger, 1951; Madoff, 1970).

The ability of L colonies to revert was sometimes dependent on how far they have been removed from contact with the inducing agent. Thus, freshly isolated strains were more prone to reversion than those which have undergone repeated serial passage on media devoid of the inducing agent (Hamburger & Carleton, 1966b). Highly alkaline media appear to promote reversion in L forms derived from Neisseria meningitidis (Roberts & Wittler, 1966).

Dienes (1970a) used a large spore-bearing bacillus (designated by him as Bacillus Y) to obtain reversion in stable L forms of Haemophilus influenzae, Proteus and Salmonella. L forms of E.coli derived from a diaminopimelic acid (DAP) auxotrophic strain reverted to the bacillary form when DAP was added to the medium (Lederberg & St.Clair, 1958). Clive and Landman (1968) have reported that by growing L colonies on filter membranes placed on agar, reversion to the bacillary state was promoted and they also stated that incorporation of crude cell wall extract into growth medium had a similar stimulatory effect on reversion. Landman and Ginoza (1961) have suggested that the infrequent reversion occasionally encountered in stable L forms of Salm. paratyphi could have resulted from a gene mutation.

Reversion of the L forms to the bacillary state can be prevented specifically by adding to the media D-amino acids (Landman & Halle, 1963), or chloramphenicol and

6-azauracil (which are known inhibitors respectively of protein- and RNA-synthesis)(Altenbern, 1963) or by transferring DNA from stable L forms to recipient and competent intact cells (Wyrick, McConnel & Rogers 1973).

IMMUNOLOGY AND SEROLOGY OF L FORMS

The serological similarities between microorganisms usually reflect their genetic relatedness; thus when the identity of L_1 organisms was first raised, one of the first characteristics to attract attention was the serological relationship between the L_1 organisms and Streptobacillus moniliformis. In one of the first studies of this kind on L forms Klieneberger (1938) classified 16 L type organisms into 7 groups by cross-agglutination tests. Included among the 16 strains were several isolates from rats as well as the so-called 'symbionts' from Streptobacillus moniliformis cultures. Serological cross-reactions between the L_1 group of organisms and Streptobacillus moniliformis were observed in these initial studies. Subsequently she demonstrated a serological difference between Streptobacillus moniliformis and its L form by agglutinin-absorption tests (Klieneberger, 1942). She demonstrated that L_1 anti-serum prepared in rabbits contained agglutinins only of one kind directed against the L_1 elements, while Streptobacillus moniliformis antiserum contained 2 kinds of agglutinins, one reacting with the L_1 elements and the other reacting with the bacillary elements. Contrary to Klieneberger's findings, Dawson and Hobby (1939b) claimed that no serological difference could be demonstrated between the L forms and

Streptobacillus moniliformis. Apparently the latter workers came to this conclusion on the basis of agglutination, agglutinin-absorption, precipitation and complement-fixation tests. Dienes (1949a) also noticed that Proteus and its L derivative were serologically identical. Dienes and co-workers (1950b) reported that in the type 3A L cultures of Proteus, titres produced by the bacterial antigen were appreciably higher than the corresponding L antigens and the agglutination reaction with the latter was characterised by the production of large floccules. In two of the three Proteus L forms they used, marked strain specificity was noted in that the L antigen of either of these reacted only with the homologous serum or with the serum produced against the corresponding parental form; in the L form of the third strain, however, no common serological relationship appeared to exist between it and its parent form, but the L form serum produced against this strain reacted significantly with the bacillary antigens of the other two strains. It also appeared from these studies that the bacillary antigens were less specific in their reactions than the L form antigens. The agglutinin-absorption tests they employed clearly demonstrated a relative deficiency of antigens in the L forms compared with the bacillary forms in two of the strains, but in the third strain there appeared to be antigens present in both L forms

and bacilli which were not common. Precipitation tests to establish serological relatedness between L forms and their bacillary phases appeared to work well with Proteus spp. but not with salmonellae (Dienes et al., 1950b). In Proteus spp. during L transformation most of the somatic antigens appeared to have been retained but the flagellar antigens were lost during such gross structural and morphological changes (Tulasne, 1951). These findings appeared to have been confirmed by Minck and Lavillaureix (1956) and Minck and Kirn (1960). The immunological identity of antigens present in the bacillary and L forms of Proteus have also been studied by Sharp and Dienes (1959). Various whole-cell bacterial and L form extracts were reactive in precipitation tests using a serum prepared against whole bacteria. Absorption and quantitative precipitation tests further confirmed the similarity of the antigens present in these two morphdogically different forms. Quantitative estimations of these antigens showed that they were present in greater amounts in the cell wall fraction than in the cytoplasmic fractions and, according to these workers, this suggested that although the somatic antigens were common constituents of both the cytoplasm and the cell wall, it was probable that they were produced in the cytoplasm but finally incorporated into the cell wall. It must be noted, however, that the

results of these two workers cannot be strictly compared with the earlier findings of Dienes and co-workers (1950b) because, while the former were using the type 3B L Proteus colonies, Dienes and co-workers were using type 3A L colonies. Weibull, Bickel, Haskins, Milner and Ribi (1967), using the gel diffusion technique, studied the serological properties of phenol extracted and trichloroacetic acid (TCA) extracted antigens in three Proteus strains and their stable L form derivatives. In two of the three strains the phenol extracts of the L forms gave well defined precipitin lines with antisera prepared against the parent bacteria as also did the phenol extracts of the bacillary antigens. The precipitin lines obtained with TCA extracts from L forms were weak denoting an antigenic deficiency in the L forms. (The trichloroacetic acid extracts from the L forms and the normal cells were chemically dissimilar.) With the third strain there was no cross-precipitation reactions between the phenol extracts of the L form and the antiserum against the parent organism nor with the phenol extracts of the bacilli and the antiserum against the L form. These findings of Weibull and associates (1967) closely parallel the earlier observations of Dienes and co-workers (1950b). As in Proteus, the L forms in

a number of salmonellae were agglutinated at a lower titre than the corresponding bacillary antigens. The specificity of the L form antigens in producing agglutinating sera was comparable with the bacillary antigens in that while some serological heterogeneity occurred between the strains, no cross reactions occurred with other species of bacteria (Weinberger et al., 1950). The somatic antigenic components in salmonellae are to some extent located in the protoplasm and protoplasmic membrane as shown by Salm.typhi protoplasts being agglutinated by sera produced against whole cells at lower titres. Furthermore, antibodies to O factors 9 and 12 were detected in antisera prepared against Salm.typhi protoplasts (Carey & Baron, 1959). It was also demonstrated by these workers that the protoplasts derived from Vi positive Salm.typhi appeared to retain Vi antigen and thus were capable of eliciting a protective response in mice. As these workers have shown, it is probable that the Vi antigen is adsorbed to the protoplast surface rather than incorporated into it because protoplasts from Vi negative strains can be made to adsorb these antigens. No major serological differences were demonstrated between Salm.typhi spheroplasts (glycine-induced) and parent bacillary forms except for a deficiency of H antigens in the glycine-induced spheroplasts (Diena et al., 1965), although this loss of H antigens is not complete.

A number of publications on the serology and immunological properties of Salmonella L forms and their revertants appear in the Russian literature. Particularly worth mentioning are the findings of Lipnicki (1958) and Kagan and Koptelova (1963). Lipnicki (1958) noted a close serological relationship between Salm.typhi bacillary forms and their L form derivatives. H antigen was shown to be completely absent in the L forms. An analysis of his results shows that in general, L antigens gave comparatively low titres compared with the corresponding bacillary antigens in the sera prepared against the bacillary forms. However, the L-antigens were agglutinated by homologous anti-L sera at titres as high as those achieved by the bacillary antigens. A similar pattern is shown in the complement-fixation tests. In haemagglutination tests, antisera prepared against L forms of Salm.typhi gave very high haemagglutinating titres with whole cell bacterial extracts , but no data were given about L suspensions. It would have been interesting to find out whether the L forms would have reacted in the same way. The immunogenicity of the L forms of Salm.typhi appears to be of a low order compared with that of the bacillary forms. Extremely large doses of live Salm.typhi L form antigens were required to elicit appreciable immune responses in mice (Kagan & Koptelova, 1963). In Pseudomonas aeruginosa serological differences between L forms and the parent

organisms appeared to vary depending on how growth of the L forms was obtained in liquid media (Bertolani et al., 1975). L forms that were transferred from solid to liquid media without gradual adaptation (Ld forms) although retaining the somatic antigens of the parent bacterium differed from the latter in that the Ld forms cross reacted with antisera prepared against two serologically unrelated strains of Ps.aeruginosa.

In the L forms that were gradually adapted to grow in liquid media (i.e. Lf and Ls forms) the Lf forms (fast growing) were strongly agglutinated by three type specific antisera none of which agglutinated the parent bacterium. The Ls (slow growing) cultures failed to agglutinate any of the antisera prepared against the bacillary forms of Ps.aeruginosa. These serological characterisations were based on slide agglutination tests only and thus no conclusive evidence ofantigenic modifications in these variant L forms can be made unless more sensitive serological tests were used to corroborate the findings. No significant differences were noted in agglutination and complement-fixation tests between smooth Brucella abortus/Brucella suis strains and their L derivatives; the L antigens gave better titres in agglutination tests but were inferior to the bacillary antigens in the complement-fixation tests and were also immunogenically inferior

(Peschkov, 1971).

Dienes (1953b) reported a serological similarity between the L forms derived from α-haemolytic streptococci and the corresponding bacillary phase on the basis of a simple agglutination test. The agglutination reactions were of a specific nature and no cross reactions were noted with other streptococcal strains, their L forms or oral strains of Pleuro-pneumonia like Organisms.

A totally different picture emerged in the serological investigations into Group A streptococcal L forms. Group A streptococci are classified mainly on the group specific polysaccharides and further typed on the basis of M antigens. It has been shown that Group A streptococcal polysaccharide is a major constituent of the cell wall (McCarty, 1952) and likewise the M protein antigens are also cell wall associated (Salton, 1953). Any loss of cell wall material would be expected to produce a significant loss of serological activity, thus the lack of the polysaccharide fraction has been shown in the L forms and protoplasts of Group A streptococci by Sharp, Hijmans and Dienes (1957), Freimer et al. (1959) and Gooder and Maxted (1961). Perhaps the most interesting phenomenon of Group A streptococcal protoplasts and L forms is the presence of the type specific M protein antigen which in the parent cocci

is located on the cell wall. Freimer et al. (1959) demonstrated that during protoplast formation and growth and to a lesser extent in the L forms, the M substance is liberated into the medium in which the organisms are growing and is not incorporated on to the limiting membranes as happens in the intact cocci. The findings of Lynn and Muellenberg (1965) indicated that Group A streptococcal L forms contained an antigenic component not present in the parent coccal forms, but these authors pointed out that the residual agglutinating activity in the L antiserum following absorption with streptococcal antigen was more likely to be the result of the streptococcal antigens being masked so preventing complete absorption. Further evidence that Group A streptococcal L forms may contain additional antigens not present in the parent vegetative forms was found in the experimental work presented by Crawford (1960). In this study it was shown that complement-fixing antibodies produced in rabbit sera following injections of either the L forms or corresponding Group A streptococci reacted with an extract prepared from the L form; in human L positive sera, however, the complement-fixing antibodies were not absorbed by normal coccal extracts or cell wall extracts but when such sera were treated with L form extract, the complement-fixing activity was completely exhausted by L extracts, denoting the presence of an antigen in the L forms

not shared by the parental forms.

Contrary to the findings of Crawford (1960) and Lynn and Muellenberg (1965), it was shown by Havliček and Havličkova (1967) that the Group A streptococci and their L forms shared a common antigenic structure but that it was probable the L forms lacked the group-specific polysaccharide. These two workers, using gel diffusion techniques, identified 4 antigens (shared by both the coccal forms and their L derivatives) which were reported to be associated with the cytoplasm and the cytoplasmic membrane and these differed in known properties from the antigens in the Group A Streptococcus. According to them the 4 antigenic components which they characterised appeared to be related to the P antigen of Lancefield (1925). Gel electrophoretic patterns of L forms derived from Streptococcus MG were almost similar to those produced by the bacillary forms (Madoff, 1970). In Group D streptococci where group specificity is largely determined by the presence of teichoic acid (Wicken, Elliot & Baddiley, 1963) and type specificity by cell wall material (Elliot, 1962), the L forms derived from them appear to lack these antigenic components (Hijmans, 1962). In Staphylococcus aureus, using the gel diffusion precipitin test, the derived L forms have been shown to lack a major cell polymer, ribitol teichoic acid (Pratt, 1966).

The presence of haemagglutinins in a fraction from Group B Neisseria meningitidis L forms was demonstrated for the first time by Crawford, Lytle and Nalewaik (1970). This fraction directly agglutinated chicken and rabbit red blood cells but not human, sheep and guinea-pig red blood cells. In a later series of experiments, Crawford, Nalewaik, Lytle and O'Connell (1971) found that the haemagglutinins extracted from the L phases of Neisseria meningitidis Group B showed haemagglutinating activity over a wider range of pH than that extracted from whole cells and the extraction of these haemagglutinins was more easily achieved from the L phase than from the parent bacterium. All animal and human sera tested appeared to possess an inhibitor to this haemagglutinin, and what was of particular interest was the finding of Crawford et al.(1971) that the decline of haemagglutination-inhibition titre in human sera against these haemagglutinins correlated with a carrier state, and loss in HI titre preceded meningococcal infection or positive Meningococcus isolation.

A number of workers have reported on the serological relationships between bacteria, their L phases and mycoplasmata. Lynn (1966) reported the existence of a common antigenic component in a Mycoplasma and diphtheroid organisms and in their L phases. Madoff (1970), on the other hand, found no such relationship

with the L forms of Streptococcus MG and Mycoplasma pneumoniae in growth inhibition tests or in the separation pattern of the antigenic components by polyacrylamide gel electrophoresis.

BIOCHEMICAL AND PHYSIOLOGICAL PROPERTIES OF L FORMS

Biochemical and physiological properties are important tools in the characterisation and identification of a microorganism. These properties have not been extensively investigated for the L phases of bacteria mainly because of the inherent difficulties associated with slow growth, osmotic fragility and the difficulty of inducing growth in defined liquid media.

Carbohydrate utilization.

One of the metabolic activities that was first investigated was the ability of L forms to ferment sugars. Heilman (1941b) reported that non-reverting L_1 organisms had the same fermentation reactions as the bacillary forms. Identical carbohydrate breakdown reactions for bacteria and their L phases have been reported in Salmonella spp. (Weinberger et al., 1950), Proteus spp. (Minck, 1952; Kandler, Zehender & Muller, 1956; Cohen, Wittler & Faber, 1968), Neisseria meningitidis (Roberts & Wittler, 1966; Cohen et al., 1968), Listeria monocytogenes (Edman et al., 1968), Streptococcus faecalis (Cohen et al., 1968) and in Streptococcus pyogenes (Cohen et al., 1968). In Pseudomonas aeruginosa the utilization of carbohydrates was generally weaker and slower in the L cultures compared with the bacillary forms (Bertolani et al., 1975). Minor differences in the ability to utilize

sugars have been reported in Staphylococcus aureus L forms and Corynebacterium strain Campo B L forms (Cohen et al., 1968).

In more detailed studies on the carbohydrate metabolism of L forms it was reported that in Group A streptococci considerable quantitative as well as qualitative differences existed between the cocci and their derived L forms in their ability to utilize various carbohydrates (Panos, 1962). These differences were most marked in the utilization of glucose and the amino sugars. Furthermore it was shown that the L forms in these studies lacked selective control of their carbohydrate utilization as shown by their increased glycolytic activity in media containing higher amounts of glucose. Smith (1964) was of the opinion that L forms may employ both the Emden-Meyerhof pathway and a cytochrome-dependent respiratory terminal pathway in carbohydrate metabolism and these being essentially similar in parent forms. In stable L forms of Proteus anaerobic utilization of glucose was found to be higher than in its parent bacillary forms (Mandel, Terranova, Sensenbrenner & Feo, 1959). Starch synthesis by L forms of Streptococcus pyogenes occurred when glucose in the growth medium was replaced by maltose (Gooder & Maxted, 1961).

Lipid metabolism.

Very little information is available on lipid

metabolism in L forms of bacteria. Large amounts of free cholesterol have been found in L cultures of Streptobacillus moniliformis (Partridge & Klieneberger, 1941), and whether this is an indication of lipid synthesis has not been conclusively proven although Partridge and Klieneberger were inclined not to think so, and did not explain how it was produced. There were indications that L forms of Corynebacterium metabolise lipids (Poetschke, 1955). Rebel, Bader-Hirsch and Mandel (1963) reported that L forms of Proteus absorbed free cholesterol from the medium but were unable to synthesise it. It has been shown that there are considerable quantitative differences in the lipid content between L forms and their corresponding bacillary phases (Vendrely & Tulasne, 1953; Smith & Rothblat, 1962; Krembel, 1963; Nesbitt & Lennarz, 1965; Cohen & Panos, 1966; Panos, Cohen & Fagan, 1966). Based on recent studies on the membrane biochemistry of Streptococcus pyogenes and its derived L form, Panos (1968) has suggested that the increases in the lipid content and the observed alteration in lipid metabolism at the subcellular level may be either an attempt by the reproducing L form to strengthen its fragile membrane or the result of direct cell wall damage or even a combination of both.

Nitrogen metabolism.

Nitrogen metabolism in Proteus and its L form

have been reported to be similar except for the inability of the latter to liquefy gelatin or decompose urea (Kandler & Kandler, 1955; Kandler et al., 1956). It had been reported earlier by Dienes (1949a) that one of the main biochemically characteristic features of Proteus spp., namely the breakdown of urea, was also a distinct biochemical feature of Proteus L forms. The production of H_2S and indole in Proteus L forms has been found to be identical with that of the bacillary forms (Minck, 1952). Cohen and co-workers (1968), who studied the biochemical reaction of L phases derived from a number of bacterial species, noted that in Proteus spp. and in some Staphylococcus aureus strains, urea hydrolysis by the bacillary forms was qualitatively similar to that produced by their L derivatives. In another strain of Staphylococcus aureus, while the bacillary forms hydrolysed urea, their L forms did not and in Corynebacterium Campo B the reaction in the L forms was variable. Phenylalanine deamination occurred both in the bacillary and L phases of Proteus mirabilis; similarly arginine hydrolysis occurred both in the bacillary and L phases of Streptobacillus moniliformis, Streptococcus faecalis, Streptoccus pyogenes and in some strains of Staphylococcus aureus.

Nitrate reduction occurred in both the L phases and vegetative forms of Proteus mirabilis and

Staphylococcus aureus. Nitrate reduction has also been reported for the L phases of Pseudomonas aeruginosa (Hubert et al., 1971). H_2S production in L forms of Streptobacillus moniliformis was noticeably increased when small amounts of sodium thiosulphate were added to the growth medium (Heilman, 1941b). Indole and H_2S production in Proteus L forms have been reported by Minck (1952). Panos (1966) in his metabolic studies on Streptococcus and its stable L form has found no gross changes in the nucleic acid composition on L transformation but the ribosomal RNA content was lowered.

Enzymatic properties.

Catalase production in Proteus L forms has been recorded by a number of investigators (Minck, 1952; Weibull & Beckman, 1961; Weibull & Hammarberg, 1962, 1963; Weibull & Gyllang, 1965; Cohen et al., 1968). Catalase activity of the L forms of Proteus was shown to be of the same order as that of the bacillary forms (Weibull & Hammarberg, 1962, 1963). In Staphylococcus aureus the derived L forms retained the capacity to produce catalase (Smith & Willis, 1967; Cohen et al., 1968). This is in contrast to the earlier findings of Weibull & Gyllang (1965) who noted loss of catalase and cytochrome activity during the L transformation of staphylococci and corynebacteria, but these were retained

in Proteus L forms. Meningococcal L forms retained the capacity to produce oxidase (Cohen et al., 1968) but in Pseudomonas aeruginosa the derived L forms lost this property (Hubert et al., 1971). Bertolani et al. (1975), however, noted oxidase production in one only of the three variant L cultures derived from a single bacillary strain of Pseudomonas aeruginosa. DNAse (Deoxyribonuclease) activity was not lost during L transformation of Staphylococcus aureus (Smith & Willis, 1967) or in phage-associated lysin-induced protoplast colonies (Freimer et al., 1959). Succinic dehydrogenase activity, being a membrane associated function even in the intact bacterial cell (Hughes, 1962), was retained in the L forms of Proteus (Weibull & Beckman, 1961; Weibull et al., 1967). Lynn (1962) compared the alkaline and acid phosphatase and inorganic pyrophosphatase activities of an L form and its parent Group A Streptococcus. The L forms possessed considerably less alkaline phosphatase activity and about an equal acid phosphatase activity compared with its parental forms. The L forms had significantly greater pyrophosphatase activity over the coccal forms. Enzymes such as gelatinase and lipase were as active in the L forms of Staphylococcus aureus as in the corresponding vegetative forms.

Extracellular enzymes, exotoxins and endotoxin production.

M Protein, haemolysin and Streptolysin S have all

been demonstrated in the L derived forms and protoplasts of Group A streptococci (Sharp et al., 1957; Freimer et al., 1959; Maruyama, Sugai & Egami, 1959; Karakawa, Rotta & Krause, 1965). Mortimer and Vastine (1967) noted the production of capsule-associated hyaluronic acid in Group A streptococcal L forms, indicating that this is not lost during L transformation. Similarly a cell-wall-associated lytic enzyme was demonstrated as a component of L forms derived from Bacillus licheniformis (Frosberg & Ward, 1972). Coagulase production in staphylococcal L forms has been observed by a number of investigators (Mattman et al., 1961; Williams, 1963; Smith & Willis, 1967). Endotoxin production by L phases of certain bacterial species is well documented. The retention of endotoxins by L form of Vibrio was shown by Tulasne and Lavillaureix (1955). Considerably less endotoxins were elaborated by the L forms than bacillary forms (Dasinger & Suter, 1962; Diena et al., 1964; Weibull et al., 1967). Phenol extracted purified endotoxins from Proteus L forms exhibited the same toxicity and pyrogenicity as the parent bacillary forms on a weight to weight basis, but the total yield was only about one third that of the normal Proteus bacillary form (Weibull et al., 1967). Kalmanson and Guze (1968) demonstrated toxic properties in chloroform extracts of the L phases of Proteus spp. suggesting that some of the toxic

substances are of a lipoidial nature. The derived L forms of Salmonella paratyphi exhibited from 5.5 yo 9 times less endotoxic activity than the parental forms (Dasinger & Suter, 1962). This toxicity was not lost by L forms even after several serial propagations. Likewise glycine-induced spheroplasts retained the Vi antigens of the bacillary forms but were only half as virulent as the latter (Diena et al., 1964).

Cary and Baron (1959) as well as Weibull and associates (1967) were of the opinion that the presence of the endotoxins in variable quantities in intact bacilli, in the L phases and in other membranous fractions suggested that these toxins were probably synthesised at the cytoplasmic membrane level and finally transported and integrated into the cell wall structures of the normal bacilli.

Vibrio cholerae L forms elaborate neuraminidase (Madoff, Madoff-Annenberg & Weinstein, 1961). The production of exotoxins by the L phases derived from Clostridial spp. appears to be a variable character. Clostridium tetani L forms have been reported by Dienes (1950) to be non-toxigenic, whereas Scheibel and Assandri (1959) maintained that these were toxigenic. The findings of Scheibel and Assandri (1959) appear to have been confirmed by Rubio-Huertos and Gonzalez-Vazquez (1960), although the latter were using glycine-

induced L forms. Clostridium perfringens L forms were reported to be non-toxigenic although haemolysin production was sometimes observed (Kawatomari, 1958).

Pigment production.

The ability to retain pigment production during L transformation has been noticed in some bacterial species while in others this function appeared to have been lost. Staphylococcus aureus L forms in appropriate media, produced pigments of a similar appearance to those of the parental form (Smith & Willis, 1967), but in L forms of Pseudomonas aeruginosa pigment production was absent (Hubert et al., 1971). In contrast Bertolani and co-workers (1975) found that production of diffusable pigments was present in all three of the L colony variants derived from a single strain of Pseudomonas aeruginosa. This apparently conflicting finding of the two groups of workers was probably due to the fact that, while the former group of investigators was dealing with the stable L forms, the latter group reported findings from unstable L forms derived from Pseudomonas aeruginosa. Pigmented strains of Serratia marcescens completely lost the ability to biosynthesise pigments during L transformation (Hubert, Potter, Kalmanson & Guze, 1969) while the converse process has been observed occasionally in Streptobacillus moniliformis (Heilman, 1941b).

Metabolic activities of different morphological elements found in L cultures.

Very little information on the physiological and related metabolic activities of the various morphological elements found in L cultures is available. The published findings of Mandel et al. (1959) and Weibull and Beckman (1961) are the most informative in this respect. Mandel and co-workers (1959) studied the respiration of various elements found in the L cultures of Proteus viz. large, small and elementary bodies (filterable elements) and whole cells in Krebs' medium and found that O_2 consumption of the small forms was the highest while the elementary bodies had almost zero values. They concluded that the elementary bodies lost the capacity to degrade glucose aerobically thus reflecting a loss of enzymatic activity. Weibull and Beckman (1961) found that elements which had diameters <0.3μm had respiration rates equal to the larger elements in Proteus L cultures, but nucleic acid synthesis was virtually absent. They concluded that the small bodies, being devoid of DNA and being unable to exhibit nucleic acid synthesis, were unlikely to be capable of reproduction.

ULTRASTRUCTURE OF L FORMS

A brief prelude on bacterial cell ultrastructure is presented before dealing with the ultrastructural anatomy of its derived forms, mainly to serve as a basis for comparing the structural changes that take place during transformation of bacteria into wall defective variants.

Bacterial anatomy studied by light microscopy does not provide much discriminating evidence for the differentiation of the various structurally altered bacterial variants other than their size and shape. The sectional profile of these altered forms, although it is not the ultimate criterion in their characterisation, provides, in conjunction with cytochemical evidence, not only the anatomical distribution and arrangement of the various macromolecules that go to form the cell wall, but also reveals the basic structure of cytoplasmic organelles and the fine structure of the nuclear apparatus.

Thin sections of bacteria viewed under high magnifications of the electron microscope show the bacterial cell wall as a multilayered structure (Kellenberger & Ryter, 1958; Beer, 1960; Glauert, 1962; Salton, 1964; Murray, Steed & Elston, 1965; de Petris, 1965; Remsen & Lundgren, 1966; Nermut, 1967; Murray, 1968). At this ultrastructural level

the differences between Gram positive and Gram negative bacteria are evident in that the cell wall in Gram positive organisms is a thick and homogeneous layer 200Å in thickness compared with a structure made up of several layers of varying thickness in Gram negative organisms (Murray, 1968). Nermut (1967) has shown, however, that Gram positive bacteria, if suitably fixed and stained, would also reveal a multilayered cell wall structure. Remsen and Lundgren (1966), working with a Gram negative bacterium Ferrobacillus ferrooxidans, compared the fine structure of the cell wall by chemical fixation and freeze etching techniques and found that chemically fixed cells showed a cell wall made up of 5 distinct layers whereas cells prepared for electron microscopy by the freeze etching method showed a three-layered cell wall complex each measuring 100Å, which they identified as (a) the outermost lipoprotein-lipopolysaccharide layer (b) the middle layer composed of globular protein attached to fibrillar mucopeptide and (c) the inner most layer being the cytoplasmic membrane studded with particles 100-120Å in diameter. They considered the freeze etching technique to be the superior method as it did not produce artifacts.

de Petris (1965), working with thin sections of E.coli, demonstrated 4 layers which he described as

the cell envelope. He distinguished the cell envelope from the cell wall and, considering it to include the cytoplasmic membrane, described it as consisting of (i) an outermost triple-layered membrane 50-55Å in thickness, which he designated as the L membrane (ii) a G layer of similar thickness (iii) a layer of low electron scattering, 40-45Å thick (M layers) and (iv) the triple-layered cytoplasmic membrane of 50-55Å thickness. The layer common to both Gram positive and Gram negative bacteria is the innermost mucopeptide layer of the cell wall (Murray et al., 1965; Nermut, 1967). This layer in the Gram positive Bacillus megaterium is given as 90-110Å thick while in the Gram negative it is relatively thinner, being estimated as 20-30Å thick (Murray et al., 1965; Nermut, 1967). The rigidity of the cell wall is derived from this mucopeptide layer (Weidel, Frank & Martin, 1960; Martin, 1963; Weidel & Pelzer, 1964). The removal of this layer would thus render a bacterial cell no longer rigid and consequently the cell would assume a shape largely determined by hydrostatic pressure exerted by the protoplastic contents on its own non-rigid limiting membrane. Whether such a structure would result in a protoplast, a spheroplast or an L form, or undergo lysis depends not only on the substrate surrounding the organism but on the manner by which the rigidity is abolished. Electron

micrographs of protoplasts reveal them as spherical bodies completely devoid of cell wall but appendages such as flagella are retained (Weibull, 1953; 1968; Thorsson & Weibull, 1958a). Another cytological aspect of protoplast formation is the gradual loss of mesosomes from Gram positive bacteria during the formation of protoplasts. These are gradually extruded into the interspaces of the cytoplasmic membrane and cell wall but completely disappear in the fully formed protoplasts (Fritz-James, 1964). While lysozyme is able to remove cell wall structures from Gram positive bacteria and present a naked wall-less protoplast, the walls of Gram negative bacteria do not permit such a degradation (Thorsson & Weibull, 1958a; Martin, 1963; Weibull, 1968). Gram negative bacteria that produce osmotically fragile spherical bodies under the combined influence of lysozyme and versene or through metabolic inhibitors of cell wall synthesis such as penicillin or glycine, have been shown to retain cell wall structures that can be seen in electron micrographs (Thorsson & Weibull, 1958a & b; McQuillen, 1958; Salton & Shafa, 1958; Martin, 1963; Hines, Freeman & Pearson, 1964; Weibull, 1968). The'protoplast-like bodies' of E.coli (spheroplasts) described by Thorsson and Weibull (1958) possessed a double-layered cell envelope, the inner layer of which constituted the cytoplasmic membrane and the outer one they regarded

as partly degraded cell wall. The 'protoplasts' in McQuillen's (1958) electron micrographs of penicillin-treated and DAP-deprived E.coli are probably spheroplasts. Penicillin-induced spheroplasts of Vibrio metchnikovi were shown to possess a double-layered membrane, the inner one being the cytoplasmic membrane (Salton & Shafa, 1958). Weibull's (1968) electron micrographs of Proteus mirabilis and E.coli spheroplasts clearly demonstrated the double-layered peripheral cell wall membrane. No differences were noted in the fine structure of glycine-induced and penicillin-induced spheroplasts of Brucella suis (Hines et al., 1964).

Three methods of examination by electron microscopy have generally been used in the study of L forms of bacteria, namely:-

(a) Whole mount examination of unsectioned L forms viewed under the conventional transmission electron microscope either directly or more usually after shadow casting or negative staining.

(b) Scanning-beam electron microscopy (SEM), a technique which permits a three dimensional perspective of a large number of L bodies at a time.

(c) Thin sectioning methods which show the internal anatomy of the various elements found in L cultures.

Conventional whole mount examination of unsectioned L forms.

The examination of whole mounts of unsectioned L forms viewed under the ordinary transmission electron microsope have generally revealed the basic elements in the characteristic arrangement as seen in preparations examined under light microscopy (Smith, Mudd & Hillier, 1948; Dienes, 1953c; Pease, 1957; Weibull, Mohri & Afzelius, 1965; Weibull, 1965). In addition, elements beyond the resolution of the light microscope have been revealed by these studies. The smallest of these bodies found in Proteus L cultures ranged between 0.15μm and 0.2μm (Dienes, 1953c), which is in agreement with the findings of Weibull et al. (1965). The occurrence of intracellular granules in some of the large L bodies has been confirmed in electron micrographs (Smith et al., 1948; Dienes, 1953c). This method of examination could not be expected to give any further useful information on the structure of L forms.

Scanning-beam Electron Microscopy.

Relatively little published information on SEM studies carried out on L forms appears in the literature. The SEM studies of Fass, Carleton, Watanakunakorn, Klainer and Hamburger (1970), Watanakunakorn, Fass, Klainer and Hamburger (1971) and Fass (1973) all indicate

that changes in the surface morphology of Staphylococcus aureus take place during transformation into wall defective forms. Unlike the parent coccal forms which on the SEM micrographs depicted uniformity of size and shape and a smooth surface appearance, the staphylococci undergoing transformation under the influence of antibodies or muralytic enzymes were generally larger and appeared to have a corrugated and "wisp-like" appearance.

Ultrathin sections of L forms.

Thorsson and Weibull (1958a, b) were the first to describe the anatomy of sectioned L elements as revealed by the electron microscope. They examined L cultures of Proteus vulgaris and found these to consist of spherical and ellipsoidal bodies of varying sizes the smallest of which occurred both as extracellular units and as intracellular granules in vesicles of the larger elements. All the elements in these L cultures were bounded by a single unit membrane. From the ultrastructural studies conducted by a number of workers it is evident that 2 main types of L elements are present in L type growth. The first consists of L elements found in stable colonies or in bacteria which form the type 3A L growth. These forms are characterised by the complete absence of cell wall and are bounded only by the cytoplasmic membrane (Thorsson

& Weibull, 1958a,b; Tulasne, Minck & Kirn, 1962; van Iterson, Ruys & Botman, 1964; Ryter & Landman, 1964; Weibull, 1965, 1968; Dienes & Bullivant, 1967, 1968; Dienes, Madoff & Bullivant, 1967, 1968; Dienes & Carlberg-Bacq, 1973). Hofschneider and Lorek (1962), however, have described a two 'unit' membrane for stable L forms of Proteus.

The second type of L forms consists of elements which, in addition to the cytoplasmic membrane, possess additional peripheral layers of modified cell wall structures and is characteristic of the type 3B L colonies of Proteus and Salmonella (Dienes & Bullivant, 1967, 1968; Dienes, Madoff & Bullivant, 1968; Delmonty et al., 1973).

The L forms of Neisseria meningitidis described by Page, Ashley and Roberts (1967) had an outer membrane in addition to the cytoplasmic membrane. Hatten, Schulze, Huang and Sulkin (1969) have described the penicillin-induced L forms of Brucella abortus as 'double membraned' structures, the inner membrane surrounding the granular cytoplasm and the outer membrane separated from the inner by a clear space. Both the inner and outer membranes were described as triple-layered 'unit' membranes. In addition, these workers have described transitional forms whose peripheral membranes were more complicated in structure than the L forms.

Membranous organelles such as mesosomes were

generally absent in L forms derived from most Gram positive bacteria and in L forms derived from some of the Gram negative species (Ryter & Landman, 1964; Weibull, 1965; Page et al., 1967) but Hatten et al. (1969) have described the presence of such structures in the 'transitional' forms in Brucella abortus L cultures. The small bodies (called elementary bodies by some workers), found both extracellularly between the larger elements as well as within larger bodies, have an organised structure and are believed to originate from the large bodies (Ryter & Landman, 1964; van Iterson et al., 1964; Weibull, 1965; Thorsson & Weibull, 1958a,b; Page et al., 1967; Dienes & Bullivant, 1967, 1968; Roberts, 1968; Dienes, Madoff & Bullivant, 1968, Hatten et al., 1969; Hubert et al., 1971). Core-like structures have been reported only in the stable L forms of Pseudomonas aeruginosa in Gram negative bacteria (Hubert et al., 1971). The differences in fine structure of L forms derived from Gram positive and Gram negative bacteria are often indistinguishable at ultrastructural level (Weibull, 1965), but more recent findings indicate that subtle differences do exist even between L forms derived from Gram positive bacteria (Schönfeld & de Bruijn, 1973).

PART II

IN VITRO STUDIES

PART II

GENERAL INTRODUCTION

The Salmonella group of organisms is particularly suited for the study of L transformation for several reasons. Organisms of this group are among the few bacterial species that produce type 3A and type 3B L colonies (Dienes, 1949a; Weinberger et al., 1950), possess a high capacity for L transformation (Terranova & de Gregorio, 1957) and, along with Proteus spp., are of the only known group in which L forms have undergone permanent changes with respect to their biological characteristics thus constituting the C type of L forms (Dienes, 1970b).

Although the first isolation of a Salmonella L form was in an English language reference, most of the work pertaining to L forms in salmonellae appear in the Russian literature. L colonies were first isolated from Salmonella typhi with the help of penicillin (Dienes, 1948a) and subsequently Dienes and co-workers (1950a) studied the effects of chemicals, antibiotics, dyes, amino acids, immune sera and complement on L induction in this group of organisms. The vast majority of the reported L inductions in salmonellae are from Salm. typhi, Salm. paratyphi, Salm. typhimurium and Salm. enteritidis, the only exception being a study

of L variation carried out by Mattman and associates (1969) involving 134 strains of Salmonella. Included in this study was a strain of Salm. pullorum. To the present author's knowledge L transformation in Salm. gallinarum has not been reported in the literature although spherical cell transformation was achieved in Salm. gallinarum by the use of penicillin (Salton & Shafa, 1958).

The present study was undertaken to investigate the following:-

(a) The effectiveness of penicillin, glycine and complement, in bringing about L transformation in Salm. gallinarum,

(b) the influence of various environmental factors on L transformation in this species,

(c) the optimum growth conditions and properties of the L forms,

(d) the factors that produce reversion and the properties of the revertants,

(e) the capacity of different strains to undergo L transformation,

(f) the filterability of the L forms,

(g) the structural alterations that take place in the cells as a result of these transformative changes as revealed by light and electron microscopy and

(h) the reproductive process in these altered forms.

A part of the present studies was also directed

towards the verification of some of the biological properties of L forms reported by various investigators. It is hoped that the findings from these studies will not only add some information on L forms in Salmonella spp. in general but also form a basis for the investigation of the role of these altered forms of Salm. gallinarum in the pathogenesis of fowl typhoid.

TERMINOLOGY

Because of the variety of names which have been used by different investigators to describe bacteria with damaged cell walls and in order to avoid confusion, it is customary nowadays in this field to define the terminology used. The following terms are used in the present investigations to denote the various cell-wall-defective bacterial variants.

L forms - these are wall-defective bacteria that exhibit considerable variations in shape, size, staining properties and cultural characteristics from normal bacterial cells and are able to propagate themselves serially as altered variants in appropriate media. The colonial growths produced by these are referred to as L form colonies, L growth or L phase growth, and the individual elements are referred to as L forms, L elements or L phase elements; the terms stable and unstable L forms are expressedly used not to connote any particular colony morphology

Spheroplasts - as used here the term will denote bacterial cells that have assumed a

spherical shape as a result of transformative changes brought about by inducing agents in a liquid medium. However, they possess the ability to replicate serially as spheroplasts.

Protoplasts - the use of this term has been deliberately restricted and where applied it will carry the meaning as defined by Brenner et al. (1958) (see p. 42).

Transitional forms - they are also referred to as transitional elements, transitional phase variants or intermediate forms. This term will denote all other variant forms not covered by the above definitions. This category generally comprises bacteria that exhibit either (a) filaments or (b) filaments with varying degrees of swelling and/or enlargement or (c) rods with gross distortions and swellings. These forms are considered to be the intermediate stages in the transformation of bacteria into L forms or vice versa.

Cell defective variants - this term is used in a collective sense to encompass all categories of the wall-defective bacteria described

earlier viz. L forms, spheroplasts, protoplasts and transitional forms.

T elements - this term has been used in these studies to designate abnormal forms found in L cultures. They occur in a variety of sizes and shapes viz. bulbous, saccular, budding and branching filamentous forms; Y shaped bodies, asteroid forms (see plate 30)*

C type L forms - this term is used to describe the elements formed as a result of permanent changes in the L forms and is intended to convey the same meaning as defined by Dienes (1970b).

Classical L colonies - there are L form colonies which show a 'fried egg type' colonial morphology and microscopically consist predominantly of L elements. These colonies generally also contain transitional forms. They do not contain any bacillary elements. L colonies could also occur as a confluent growth without a central core and these are referred to as patchy granular L type growths or confluent L growth.

Heteromorphic L colonies - these are L colonies in

* See Volume 2

which there are large numbers of L elements and transitional elements in which the latter constitute more than 50 per cent. of the total elements present. The term is also used when there are many bacillary elements in a growth which otherwise conforms to the definition of an L colony. Heteromorphic L colonies may occur as discrete colonies having a 'fried egg' colonial appearance in which case they are referred to as classical heteromorphic L colonies. Heteromorphic L colonies may occur as a confluent growth often indistinguishable macroscopically from a confluent bacillary growth.

Revertant type growth - this term is used to denote growth in which the predominant elements are bacilli with small to moderate numbers of transitional elements and very few L elements.

Also used in this study are a variety of terms which are non-commital in nature and are purely intended to convey a descriptive meaning and these are defined when used.

MATERIALS AND METHODS

BACTERIAL STRAINS

The following strains of Salmonella gallinarum were used in these studies:

9S - a standard laboratory smooth strain

9R - a standard laboratory rough variant

Ipoh 131/71 - a smooth strain, isolated from chicken egg

Ipoh 154/71 - a smooth strain, isolated from chicken egg

Ipoh 784/71 - a smooth strain, isolated from clinically affected chicken

Ipoh 846/71 - a smooth strain, isolated from chicken egg

Ipoh 892/71 - a smooth strain, isolated from chicken egg

Ipoh 595/72 - a smooth strain, isolated from clinically affected chicken

Ipoh 1236/72 - a rough strain, isolated from chicken egg

Ipoh 37/74 - a rough strain,isolated from chicken egg.

Strains 9S and 9R were received from Professor A. Buxton and, being standard laboratory strains, they have been subcultured many times on solid media. The remaining 8 strains are field isolates of more recent origin which have undergone minimal passages on agar. These 8 strains were received from the Director, Veterinary Research Institute, West Malaysia. On receipt all strains were lyophilised and working cultures were drawn from the organisms maintained on Dorset egg slants or nutrient agar slants. The term strain as used in

this presentation does in no way imply a genetic difference between the various isolates. As all the 8 field strains were received midway through the course of this work, they were not investigated to the same extent as the standard laboratory strains.

MEDIA

Routine bacteriological media.

These media included peptone water, nutrient broth, nutrient agar, 5 per cent. sheep blood agar, 5 per cent. horse blood agar, MacConkey's agar, Dorset egg medium (with no added dyes) and Selenite broth. Unless otherwise mentioned these media were prepared by the methods described by Cruickshank (1965).

The routine bacteriological media were used (a) for obtaining yields of normal bacillary cells to serve as the primary inocula for L induction media and biochemical characterisation media, (b) for performing part of the characterisation tests of the normal bacillary growth and the revertants obtained after L transformation, (c) for obtaining reversion of L forms and (d) for the purpose of identifying abnormal growth or contaminants.

The routine media were also used for growth curve studies during the initial part of the work. Nutrient agar slants and Dorset egg slants were used for maintenance of working cultures.

Media used for biochemical characterisation tests.

Unless otherwise stated the media used were those recommended by Cowan and Steel (1965).

Special media.

These were used exclusively for the induction, propagation and maintenance of L cultures. Most of

the media described are those advocated by different investigators and some of them were modified to study the effect of various media components in bringing about L transformation. In some instances modifications were necessary as certain commercial brands were not available locally, but in every case they were substituted with like substances from different commercial sources. Unless otherwise stated, all special media were sterilised by steam autoclaving at 121°C for 15 minutes.

(i) Alexander Jackson Medium (AJA medium):

The medium used is a modification of the one described by Alexander-Jackson (1954) with the following composition:

Digest broth	1.0 L
Myosate (BBL - BioQuest Division)	3.0 g
Gelysate (BBL - BioQuest Division)	3.0 g
Trypticase (BBL - BioQuest Division)	3.0 g
Phytone (BBL - BioQuest Division)	3.0 g
Yeast extract (Difco)	10.0 g
Sucrose (BDH)	150.0 g
Glucose (BDH)	5.0 g
Glycerol (Sigma)	40.0 ml
$MgSO_4.7H_2O$	2.0 g
Agarose (Sigma)	3.0 g
	pH = 7.4

This comprised the standard AJA medium. To study the effects of various medium components on L induction the following modifications were carried out:

MC medium - AJA medium with 0.15 per cent. calcium chloride added.

AJA (E) medium - AJA medium containing double the amount of sucrose.

NAJA medium - consisted of equal parts of AJA medium and nutrient agar.

AJB medium - this is a broth medium having the same composition as AJA medium without agar or other gelling agents. AJB was used extensively for maintaining and propagating L phases in liquid media.

AJA medium - with glycerol omitted.

AJA medium - with magnesium sulphate omitted.

(ii) MAJA medium.

Digest broth	1.0 L
Myosate (BBL BioQuest)	3.0 g
Gelysate (BBL BioQuest)	3.0 g
Phytone (BBL BioQuest)	3.0 g
Sucrose (BDH)	150.0 g
$MgSO_4.7H_2O$	0.5 g
$CaCl_2$	1.5 g
Agarose (Sigma)	4.0
	pH = 6.7

Agar concentrations - to study the effects of agar concentration on L induction, the concentration in the standard AJA medium was varied from 0.3 per cent. to 0.6 per cent. In a few experiments Difco Noble agar was substituted for the agarose.

pH - the pH of the AJA medium was also varied at 6.7, 7.2 and 8.1 to study the effects of hydrogen ion concentration on L induction and reversion.

Glucose - glucose was added either separately (as a 10 per cent sterilised by filtration through a Seitz filter grade 5) after all the other components had been sterilised by autoclaving at 121°C for 15 minutes, or together with the ingredients of the AJA media to find out if any L inhibitor substances were formed by the latter technique.

(iii) Makemson & Darwish medium (M medium).

This was prepared as described by Makemson and Darwish (1972) except that the 4 per cent. Difco beef heart infusion was replaced with beef heart digest broth.

The composition of the medium used was as follows:

Digest broth (beef heart)	40.0 ml
Glucose	8.0 g
Sucrose	200.0 g
$MgSO_4.7H_2O$	0.5 g

$CaCl_2$	1.5 g
Agar (Difco)	10.0 g
Distilled water added to make 1L	
	pH = 6.7

(iv) Modification of Medill-O'Kane medium (1954)

(MKS medium)

Composition: (Basal media)

+Glucose	1.0 g
Sodium lactate	2.0 g
K_2HPO_4	0.9 g
KH_2PO_4	0.1 g
*Salt mixture	2.0 g
Nicotinamide	1 µg/ml

*Salt mixture	per Litre
$MgSO_4.7H_2O$	40.0 g
NaCl	2.0 g
$FeSO_4.7H_2O$	2.0 g
$MnSO_4.4H_2O$	8.0 g

+Glucose was added separately as 10 per cent. solution which had been sterilised by Seitz filtration.

When a solid medium was desired a 2.2 per cent. solution of agar in sterile distilled water was added to an equal volume of the basal medium to give approximately 1.1 per cent. agar concentration. Vitamin-free Casamino acids (Difco) was added to give a final

concentration of 0.04 per cent. in the solid medium.

(v) K medium.

This was a modification of the medium described by Kawakami, Ishibashi, Mitsuhashi, Sakaino and Fukai (1970) for the isolation of Salmonella L forms.

Bacto-agar (Difco)	7.0 g/litre
Bacto-peptone (Difco)	20.0 g/litre
Yeast extract (Albimi Lab.)	1.0 g/litre
KH_2PO_4	0.035 M
NaCl	0.06 M
$CaCl_2$	0.01 M
$Mg\ Cl_2$	0.01 M
Glucose	0.01 M
Sucrose	0.35 M
	pH = 7.4

Primary inocula in liquid media were obtained by growing the bacillary forms in peptone water, nutrient broth on AJB without serum.

(vi) Hypertonic nutrient broth medium.

This is ordinary nutrient broth in which the salt concentration has been increased to 0.5M NaCl. This medium was used to test the ability of L forms to propagate in liquid media.

Preparation of primary inocula

To obtain primary inocula in liquid media the bacillary forms were grown in 9-10 ml. of one of the

following.

1. Peptone water.
2. Nutrient broth.
3. AJB.

Solutions for harvesting and suspending L forms.

The suspensions of L forms were made in AJB medium or 6 per cent. NaCl broth.

Serum.

Horse serum. Blood withdrawn from healthy horses was allowed to clot. After the serum had separated it was pipetted off, centrifuged to remove cells and sterilised by filtration through a Seitz filter. It was then dispensed in 20 ml amounts in Universal bottles and stored at -20°C. Prior to use it was thawed out and inactivated at 56°C for one hour in a thermostatically controlled water bath. Serum was added to the melted induction medium at a temperature of 45-50°C. Both haemolysed and non-haemolysed horse sera were used. Unhaemolysed horse serum was also added to AJB medium for maintenance and propagation of L forms in liquid media.

Rabbit serum. Serum was obtained from healthy rabbits which had been previously screened for Salmonella antibodies and found to be negative. Only non-haemolysed samples were used. Rabbit serum was sterilised, inactivated and used in the same way as horse serum.

All sera used were screened for sterility and

Mycoplasma growth on appropriate testing media. Serum was incorporated into the induction media to give a final concentration of either 10 or 20 per cent.

Antigenic typing.

(i) Antisera.

Salmonella diagnostic sera* were used for serotyping of parent and revertant strains of Salmonella gallinarum.

Type	Titre
Polyvalent H (specific and non-specific)	1:400
Polyvalent O (Group A-G)	1:80
Specific factor 9	1:320

(ii) Method.

Test colonies were emulsified in a drop of saline on a microscope slide and a drop of the typing serum added and mixed. The reaction was considered to be positive if the mixture showed a granular or flaky agglutination within 2 minutes of adding the typing serum.

(iii) Agglutination in acriflavine solutions.

Solutions of acriflavine were prepared in distilled water to give dilutions of 1:100 and 1:500. The ability to agglutinate was assessed by mixing a loopful of culture with a drop of the acriflavine solution placed on a microscope slide. The slide was rotated gently and type and degree of agglutination recorded within

*Wellcome Research Laboratories, England.

60 seconds. Organisms that agglutinated in acriflavine at 1:500 dilution were considered as rough strains. Colonies emulsified in distilled water served as controls.

Characterisation tests:

All biochemical characterisation tests were carried out by the methods recommended by Cowan and Steel (1970). All the 10 parental strains of Salm. gallinarum and the revertants arising from the derived L forms were studied for their morphological, cultural and serological properties. All cultures in these characterisation tests were incubated at 37°C unless otherwise specified.

The cell morphology and staining reactions of all the 10 parent strains and revertants arising from L colonies were examined in nutrient agar cultures grown at 37°C for 16-18 hours. The cell morphology of some of the strains were studied on cultures incubated at 30° and 37°C for varying periods of time. Six- and 18-hour nutrient broth cultures of all revertant and bacillary forms were examined for abnormal morphology under the phase contrast microscope.

(i) Colonial morphology.

Colonial morphology of normal bacillary forms and revertants were studied on nutrient, blood and McConkey agar plates which had been incubated at 37°C for 18 hours. Horse and sheep blood agar were used to detect haemolytic properties.

(ii) Growth in liquid media.

All parent and revertant strains were studied for growth characteristics by growing them in nutrient broth for 6 and 18 hours.

(iii) Motility.

This activity was determined by microscopic examination of 'hanging drop' preparations of 6-hour nutrient broth cultures incubated at 37°C.

(iv) Catalase production.

The organism to be tested was grown on a nutrient agar slant for 18 hours at 37°C and the catalase activity determined by running a few drops of H_2O_2 (10 volume) down the slant. The reaction was considered positive if the evolution of gas bubbles occurred within 2 minutes of adding H_2O_2 and delayed if it appeared after 2 minutes but before 5 minutes.

(v) Oxidase activity.

Pieces of Whatman's* No. 1 filter paper 10 mm. x 40 mm. were impregnated with a few drops of freshly prepared 1 per cent. aqueous solution of tetramethyl-p-phenyldiamine solution and placed in a clean glass petri dish. A loopful of culture taken from an 18-hour nutrient agar culture was smeared across the impregnated filter paper by means of a glass rod. Production of a dark purple colour within 10 second was recorded as positive

*W.& R. Balston Ltd., England.

(vi) Citrate utilization.

Modified Koser's citrate medium was used to detect the ability of all parent and revertant strains to utilise citrate as the sole carbon and energy source and ammonium salt as the nitrogen source. This medium was inoculated very lightly with agar-free colonies picked up carefully from 24-hour nutrient agar cultures. Growth was indicated by development of turbidity in the inoculated medium.

(vii) Liquefaction of gelatin.

Deep nutrient gelatin was inoculated by stabbing the medium with colonies picked up on a straight inoculating wire. The medium was incubated at 37°C and examined every 3-5 days for liquefaction by placing it in a cold room (4°C) for 2-3 hours.

(viii) Gluconate oxidation.

The organisms were grown for 48 hours at 37°C in approximately 10 ml of gluconate broth. At the end of the incubation period 1.0 ml of Benedict's solution was added and the culture boiled in a waterbath for 10-15 minutes. A positive reaction was indicated by the formation of a brown, orange or tan precipitate.

(ix) H_2S production.

Production of hydrogen sulphide was detected by inserting a previously dried sterile lead acetate paper strip (5 mm x 50 mm) between the cap and McCartney bottle

containing nutrient broth inoculated with a few drops of a 6-hour nutrient broth culture of the test strain. Blackening of the lead acetate paper was indicative of H_2S production. Depending on the intensity of the blackening of the paper the results were recorded as strong or weak.

(x) Indole production.

The test organism was grown in peptone water at 37°C for 48 hours. Indole was detected by adding 0.5 ml. of Kovac's reagent to the peptone water culture which was shaken for 1 minute and allowed to stand. The appearance of a red or pink colour in the top layer was indicative of indole production.

(xi) Growth in KCN broth.

KCN broth was inoculated with agar-free colonies from a 24-hour nutrient agar culture of the test organism. The medium was inoculated very lightly and incubated at 37°C. Positive reaction was indicated by increase in turbidity of the media after 24 hours' incubation. Basal medium with no KCN inoculated in the same way served as a control.

(xii) Combined malonate utilization and phenylalanine deamination test.

Malonate-phenylalanine deamination medium was inoculated with a colony from a 24-hour nutrient agar

culture of the test organism and incubated at 37°C for 24 hours. The development of a blue colour denoted utilization of sodium malonate. The phenylalanine deaminase test was conducted on the same medium by acidifying it with 0.1 ml + 0.2 ml of 0.1 N - HCl and adding 0.2 ml of 10 per cent. $FeCl_3$ aqueous solution. The mixture was shaken. A positive reaction was denoted by a transient green colour in the medium.

(xiii) Nitrate reduction.

Nitrate broth was inoculated with a few drops of 6-hour nutrient broth culture of the test organism, incubated at 37°C for 5 days and then 0.5 - 1.0 ml of 0.8 per cent. sulphanilic acid in 5N acetic acid was added followed by the same volume of 0.5 per cent. α-Naphthylamine in 5N acetic acid. The appearance of a red colour was indicative of the presence of nitrites. If a negative reaction was obtained a small amount of zinc dust was added to the medium and allowed to stand. The development of a red colour after the addition of the zinc dust was indicative of the presence of free nitrate in the medium. The absence of red colour indicated that the nitrate had been reduced further than nitrite.

(xiv) Nitrite reduction.

Nitrite broth was inoculated from a 6-hour nutrient broth culture and incubated for 7 days. The same reagents

used in nitrate reduction tests were also used in similar amounts in this test, but a red colour denoted the failure of the organism to reduce nitrite.

(xv) Methyl-red (MR) test.

An MR/VP medium was inoculated with a few drops of a 6-hour nutrient broth culture and incubated at 37°C for 2 days after which 2-3 drops of methyl red solution was added. The production of a red or orange colour was recorded as a strong or weak positive reaction respectively.

(xvi) Voges-Proskauer (V-P) test.

After the completion of the M-R test, 0.6 ml of 0.5 per cent. -naphthol solution and 0.2 ml of 40 per cent. KOH aqueous solution were added. The bottle was shaken, allowed to stand and examined at the end of 15 minutes and 1 hour. A positive reaction was denoted by the appearance of a red colour.

(xvii) β-galactosidase activity.

Peptone water containing O-nitrophenyl-β-D-galacto-pyranoside (ONPG) was used for this test. The medium was inoculated with a few drops of 6-hour nutrient broth culture of the test organism and incubated at 37°C for 24 hours. β-galactosidase activity was demonstrated when the originally colourless or faintly yellow medium developed an intense yellow colour.

(xviii) Urea hydrolysis.

A Christensen's urea agar slant was heavily inoculated with the test organism and incubated at 37°C. It was examined daily for 7 days. A positive colour was denoted by the development of a purple-pink colouration.

(xix) Amino-acid decarboxylase test.

The ability of the parental and revertant strains of Salmonella gallinarum to decarboxylate the following amino acids were tested - L-lysine, L-ornithine and L-arginine. One per cent. solutions of these amino acids were incorporated into the basal medium and distributed into small tubes (5 mm x 60 mm). A control set with no amino acid served as control. All the media were layered with sterile liquid paraffin to a height of 5 mm. These media were inoculated with colonies picked up with a straight nichrome wire from 24-hour nutrient agar growths of the test organism. The inoculated media were incubated at 37°C. The appearance of a violet colour was indicative of decarboxylation.

(xx) Carbohydrate breakdown.

Sugar fermentation tests were carried out in a medium described by Cruickshank (1965, p. 813-815) to which slight modifications were made. The medium used in these studies consisted of 1.0 per cent. peptone water enriched with 10 per cent. Lab. Lemco broth (peptone

10g/L, Lab. Lemco powder* 7.5g/L and sodium chloride 5g/L) with 2 per cent. bromothymol blue solution (0.2 per cent.) added as indicator and 0.5 per cent. of the fermentable carbohydrate. These media were dispensed in 5.0 ml amounts in bijou bottles and were inoculated with 6-hour broth cultures of parent and revertant strains. The inoculated media were incubated at 37°C.

In all the above tests, except where otherwise mentioned, all media were at first examined daily for a period up to 3 weeks, but, as no marked changes were observed after 14 days, subsequent observations were not extended beyond 2 weeks. All biochemical and other characterisations were duplicated. In some instances media inoculated with revertant and parent colonies were incubated at 37°C and 30°C.

In the case of L organisms only 2 isolates were subjected to some but not all of the characterisation tests. The growth characteristics, cell morphology and staining reactions of these L organisms were studied on hypertonic induction media only.

Characterisation tests for L forms.

(i) Motility.

The organisms obtained from the solid medium were suspended in hypertonic media and warmed in an incubator for 1 hour before being examined microscopically for motility

* Oxoid Ltd., U.K.

by the hanging drop method.

(ii) Catalase test.

The organisms grown on hypertonic medium were removed free of agar particles and placed on a clean microscope slide on to which a drop of H_2O_2 (10 volume) was added. The development of gas bubbles indicated a positive reaction.

(iii) Oxidase test.

This was carried out as indicated for revertant and parental strains.

(iv) Decarboxylase test.

This was carried out as indicated for revertant and parental strains.

(v) Carbohydrate breakdown.

The osmolarity of liquid media containing sugar was increased by the addition of a 40 per cent. solution of sodium chloride to give an approximate equivalent of a 0.5M solution. The osmolarity of other liquid media used for biochemical characterisations of L forms was also increased in the same way.

All characterisation media were inoculated with L colonies washed and suspended in 0.5 M NaCl unless otherwise specified. Media inoculated with L organisms were incubated at 30°C. During incubation periodic

checks were made to see that the characteristic L type growth was proceeding.

DETERMINATION OF LAG, EXPONENTIAL AND DECLINE PHASES OF STRAIN 9S

This was determined by inoculating 4x 9.9 ml of peptone water with 0.1 ml of an overnight broth culture of strain 9S of known titre. The inoculated media were incubated at 37°C and at periodic intervals samples from each bottle were drawn out and pooled. After making suitable dilutions, viable counts were made both by the streak and pour plate methods. Only counts from the pour plates were recorded and counts from three plates were averaged. The streak method was employed to check the results obtained by the pour plate method and as an alternative in the event of no growth or gross contamination occurring in the pour plates.

Two such determinations were made on separate occasions. On the basis of the experiments it was concluded that 9.9 ml amounts of peptone water inoculated with approximately 0.1 ml of nutrient broth culture containing $1x\ 10^7$ cells and incubated at 37°C would be in the exponential phase between the 4th and 8th hours of incubation. Samples drawn before the 4th hour were considered to be in the lag phase or in the stage of slow multiplication. The culture was considered to have passed the stage of exponential growth after the 8th

hour of incubation. These determinations were carried out to find out whether cells in a particular metabolic or physiological state are more prone to L transformation under the influence of an inducing agent.

DETERMINATION OF CELL CONCENTRATIONS

a) Cell concentrations were determined photoelectrically in a portable EEL* colorimeter. Cells were obtained from an actively growing 6-hour peptone water culture of strain 9S. The cells were centrifuged and washed twice in sterile saline, and suitable dilutions were made in normal saline for obtaining EEL readings. A standard curve was obtained by plotting EEL readings against the average of triplicate viable counts. Subsequent cell content determinations were obtained by interpolating EEL readings against viable counts.

b) The approximate number of cells in a given volume of liquid medium was also obtained by comparing the opacity of the bacterial suspension with standard Brown's opacity tubes**. This method was not generally used except for standardisation of antigens in the agglutination tests.

PREPARATION OF IMMUNE SERA

Experimental animals

Four large New Zealand White rabbits were used. Three were immunized and one served as a donor for

*Evans Electroselenium, Essex. **Wellcome Foundation Ltd., U.K.

non-immune serum. Prior to use they were kept under observation for a period of 4 weeks, during which time blood samples were taken at 2 week intervals to check for Salmonella antibodies. To ascertain that none of them were 'carriers', faecal cultures were screened for salmonellae by the usual bacteriological procedures.

Preparation of antigen for immunization of rabbits.

Somatic O antigens were prepared according to the method of Schlecht and Westphal (1967). Salm. gallinarum strain 9S (smooth strain) was inoculated on several nutrient agar slants and incubated at 37°C for 24 hours. The cells were harvested and the growth from the different slants pooled and checked for purity by making a Gram's stained smear and also by subculturing on to routine bacteriological media that were examined the following day for the presence of any other Gram negative growth. The pooled cells were lightly centrifuged to sediment agar particles and the supernatant decanted into sterile McCartney bottles and centrifuged at higher speeds to sediment the cells. The cells were washed twice and resuspended in approximately 10.0 ml normal saline. The suspension was then steamed at 100°C for 3-4 hours. The cells were then separated, washed twice and resuspended in 10 ml of normal saline. Sterility of the heated antigen was checked on blood agar plates.

The antigen was dispensed in 2.0 ml amounts and stored at $4^{o}C$. Rabbits were bled for pre-immunisation sera and then inoculated intravenously with 0.3 ml of the boiled suspension containing 1.2×10^{10} cells/ml. The second and third immunising doses (0.5 and 1.0 ml respectively) containing 6×10^{9} organisms per ml were also given intravenously. The injections were spaced at 4 days intervals. Rabbits were bled 4-7 days after the last injection. When more immune serum was required a booster dose (1 ml of 6×10^{9} cells per ml) was given intravenously and the animal was bled 4-7 days later. One rabbit died during the course of immunization.

Determination of titre of the immune sera.

The titres of the immune sera produced in the rabbits were determined by the tube agglutination test. Doubling dilutions of serum in 0.85 per cent. sodium chloride were prepared in disposable agglutination tubes (2" x $\frac{1}{4}$") in 0.5 ml. volumes starting at a dilution of 1 in 10 up to 1 in 2560. The antigen for the tube agglutination test was prepared by growing the organisms on several nutrient agar slants. The cells harvested in a small volume of saline (1.0 ml) were mixed with 40.0 ml of absolute alcohol and heated at $50^{o}C$ in a water bath for 30 minutes. After centrifuging, the cells were resuspended in saline and the density of the antigen was adjusted to Brown's tube no. 3. This

antigen was added to the diluted sera in 0.5 ml. amounts. The test samples were incubated at 37°C for 24 hours. Appropriate controls were also set up. The end point was taken as the highest dilution of serum showing complete agglutination.

INDUCING AGENTS

Penicillin.

Penicillin was the most extensively used inducing agent in these studies. Crystapen*, a commercial brand of Benzylpenicillin Sodium (BP) was used. Penicillin solutions were made using sterile distilled water. Concentrated solutions were held at 4°C for not more than 5 days unless otherwise stated. Penicillin was used in a number of ways to bring about induced cell transformation. It was incorporated into the media in one of the following ways:-

(i) Penicillin was added to growing liquid cultures and, at periodic intervals following such treatment, the incubated samples were subcultured on to penicillin-containing induction media as well as on to hypertonic media not containing penicillin. When used in this manner, penicillin was usually added to the liquid cultures in concentrations ranging from 1,000 to 40,000 units per ml of the medium.

(ii) Penicillin was incorporated into the solid induction medium in concentrations ranging from 40 units to

*Glaxo Laboratories Ltd., (U.K.)

4,000 units per ml of medium. The penicillin was added into the molten medium after cooling to 45-50°C. These were used as primary induction media as well as the media for propagation and maintenace of L forms.

(iii) Penicillin was diffused into the medium. When this technique was used the induction media were dispensed in 50 mm and 90 mm petri dishes to a depth of 8-10 mm. Two methods were used to set up a penicillin gradient in induction media:

(a) In the first method a circular core of agar 5 mm in diameter was removed from the centre of an agar plate containing solidified induction medium and a known amount of penicillin was instilled into the well after sealing with a few drops of the molten agar to prevent the seepage of penicillin between the plate and bottom of the agar layer. During the early part of these studies an oblong trough measuring 5 mm x 10 mm was cut from the agar at one end of the plate and penicillin was instilled into it in the same way. Small plastic petri dishes (50 mm) were used when penicillin was diffused in this manner. Penicillin was also diffused from the flIor of porcelain cylinders. Hollow porcelain cylinders measuring approximately 6mm x 10mm which had been previously sterilised were picked up with a pair of sterile forceps and heated over the bunsen flame. When one end of the cylinder was sufficiently heated it was placed in the centre of the

medium contained in large (90 mm) plastic petri dishes. The heated porcelain cup when placed properly sat firmly with a bottom floor of agar. Into this well an aqueous penicillin solution was placed which diffused out into the surrounding agar from the bottom agar floor of the porcelain well. In some of the earlier experiments, penicillin was made up in 0.5M sodium chloride solution which was then instilled into the wells.

(b) The second method of diffusing penicillin within the medium was by putting up penicillin gradients, as described by Szybalski (1952). By this method the penicillin concentration was distributed in a graded manner along the horizontal axis. Large (90 mm) petri dishes were tilted and about 20 ml of nutrient agar poured to cover the bottom of the plate. When the agar was solidified the plate was placed flat on an even surface and 20 ml of molten agar containing penicillin was overlayered on the solidified nutrient agar layer. The net effect of this modified method is to produce at one end of the plate an area of high penicillin concentration and at the opposite end an area of low penicillin concentration.

In all penicillin diffusion techniques the concentration of penicillin used ranged from 80 units to 80,000 units penicillin contained in 0.1 ml. of distilled water. Penicillin solutions were also used

to wash agar blocks to remove surface growth and also to make suspension of L colonies growing within the agar. Penicillin was also added in known amounts for induction, maintenance and propagation of spheroplasts and L forms in liquid media.

Semisynthetic penicillin.

In a small number of experiments ampicillin (Penbritin* injectable powder) was used as an inducing agent. It was also used to suppress bacillary growth.

Glycine.

A laboratory grade glycine (Analar**) was used. This was incorporated into AJA medium to give a final concentration of either 1.5 per cent. or 3.0 per cent.

Immune sera.

The ability of immune sera with high (1:1280 and above) and low (<1:640) titres as well as non-immune sera to induce L formation was tried. These sera were only incorporated into standard AJA medium in a molten state at 40-45°C. The concentration of immune and non-immune rabbit sera in the media did not exceed 10 per cent.

Complement.

Guinea-pig complement used in these studies had been preserved in borate-buffer sorbitol by the method of Richardson (1941) and then lyophilised. For use

*Beecham Veterinary Products, England.
**British Drug Houses Ltd., U.K.

the complement was reconstituted 1:8 in sterile distilled water. This was equivalent to 1:10 of guinea-pig serum.

Combined effect of inducing agents.

Penicillin in varying concentrations was added to media containing either 1.5 or 3 per cent. glycine. Similarly the effect of penicillin and glycine in AJA medium with double strength sucrose was tested for synergistic effects.

Haemolysed and non-haemolysed serum.

Haemolysed and non-haemolysed horse sera were inactivated at 56°C in a water bath and were separately added to AJA medium to study the effect of lysed sera in inducing L transformation. Uninactivated sera were also used.

INCUBATION

Cultures were incubated at 30°C and 37°C both aerobically and anaerobically to investigate whether the temperature of incubation and the gaseous environment were critical factors in the development of L forms and also whether these physical influences in any way hastened or slowed the process of reversion of L forms to the bacillary state.

CONCENTRATION OF LIQUID CULTURES AS INOCULA ('CONCENTRATED PW CULTURES')

All liquid culture inocula were incubated at 37°C unless otherwise specified. Liquid culture inocula were concentrated by centrifuging the incubated cultures and resuspending the cells in one-tenth of the original volume of PW water.

CULTURING METHODS

Liquid cultures, washings of surface growth and suspensions of agar colonies growing within the agar were subcultured either by the pour plate method or by the spread method. In the former method the molten medium was cooled to 45-50°C before adding the inoculum and then gently rolled to distribute the inoculum evenly after which it was poured into the petri dishes. In the second method a few drops of the inoculum was placed on the agar surface and spread evenly on the surface either by tilting in various directions or by using a nichrome wire or bent glass rod as a spreader. Nichrome wire and glass rods were used particularly when the induction medium was hard in order to cause a break of the agar surface to facilitate the growth of L elements into the agar. Subculturing of L colonies and other colonial variants from one solid medium to another was accomplished by cutting out under aseptic conditions agar blocks

(5 mm x 8 mm) containing the surface growth and transferring them face downwards across to the fresh medium. The agar block so transferred was left on the new medium and moved to a new site at periodic intervals. Subcultures of agar growth to liquid media were carried out by excising fragments of agar bearing colonies from the plate and dropping them aseptically into liquid media.

PROVISION OF MOISTUREDURING INCUBATION

In a few of the experiments excess surface moisture of the plates was removed by drying the plates before they were inoculated. During prolonged incubation plates were suspended on a wire gauze in a glass desiccator jar containing a small amount of sterile distilled water at the bottom.

FILTRATION

The following types of membrane filters were used in these studies:

*Sartorius membrane filters - 0.45µm and 0.6µm A.P.D.

**Millipore membrane filters - GS 0.22µm, HA 0.45µm, AA 0.80µm and RA 1.2µm A.P.D.

The filterability of the L phase was assessed by the following methods.

(i) Growth of L phase and associated type colonieswas suspended in 10-12 ml of AJB medium and a uniform

*Sartorius Membrane filter GMBH, West Germany.

** Millipore Ltd., U.K.

suspension was made by shaking. Broth cultures were filtered directly. The fluid to be filtered was drawn up into a sterile 10cc polypropylene syringe to which was then fitted a pre-sterilised Millipore unit containing a 13 mm filter membrane of the appropriate A.P.D. The filter unit had been assembled and sterilised according to the manufacturers instructions. Gentle pressure on the plunger of the syringe forced the fluid to be filtered through the membrane at the rate of 1 drop per 10-15 seconds and the filtrate was allowed to drop directly on to appropriate culture media. Following filtration the filter units were dismantled andthe membrane filters visibly inspected for leaks, cracks and other physical defects. Normal bacillary cultures served as controls.

(ii) Sterilised Millipore membranes 13 mm in diameter and Sartorius membrane filters cut into 10 mm squares and sterilised were placed on AJA medium (with and without penicillin). Cubes of agar measuring approximately 5 mm bearing L type growth were excised from the growth medium and carefully transferred to medium with filter membranes. The blocks were placed facing downwards in direct contact with the membranes alone. The inoculated plates were returned to the incubator and after varying periods of incubation were checked for growth beneath the filter membranes.

EXAMINATION OF CULTURES FOR GROWTH

(i) Agar plates were examined for growth both directly and microscopically with x5 and x10 objectives in a binocular microscope.

(ii) Growth in liquid media was detected by an increase in the turbidity of the inoculated medium and by examination of wet unstained films by phase-contrast microscopy with a x100 objective.

Agar plates showing no growth were incubated and inspected for a minimum period of 14 days before they were considered as negative. Liquid media showing no increases in turbidity were retained for at least 30 days before they were considered as negative.

(iii) L colonies giving a typical "fried-egg" appearance were examined using an ordinary light microscope with the condenser lowered and the objective displaced in an 'off clicked' position as suggested by Muelas and Ales (1973).

STAINING METHODS AND PROCEDURES

Gram staining.

Routine smears of broth and agar cultures of normal bacilli and altered variants were stained by Lillie's (1928) modification of Gram's method.

Dienes' colony staining method.

Staining of colonies in situ was done by slight

modifications of the methods recommended by Dienes (1967b, 1970b). Initially cultures on plates were fixed with formalin vapour by exposing the cultures to pieces of filter paper soaked with formalin solution in the cover of the petri dish. The petri dishes were sealed and left at room temperature. Fixation periods were varied from 3 hours up to 7 days. In the later part of these studies fixation of colonies by formalin vapour was omitted and colonies were stained without fixation. To prepare the growth for staining, thin agar slices were cut from the surface of excised agar blocks. This was done as follows:-

Agar blocks (5mm cubes) bearing colonies were cut out and placed on a clean microscope slide with the colonies uppermost. A second slide was held vertically on one side of the agar block to give it support. With a razor blade a thin slice about 1 mm in thickness was obtained by slicing the agar in a horizontal plane with a continuous sliding motion throughout the length and breadth of the agar block. The thin slice was lifted from the razor blade carefully and placed with the culture facing the glass on prestained coverslips.

The coverslips bearing the thin slices of agar were placed in a petri dish and a piece of Whatman's No. 1 filter paper, 20 mm. x 40 mm., lightly moistened, was applied directly to the agar. An unmoistened piece

of filter paper of the same size was then applied on the first piece. These papers were weighted down with two ordinary microscope slides and the petri dish was placed in a microscope illuminator fitted with a 60 watt bulb. The lid of the petri dish was replaced and kept under the heat of the bulb for 15-30 minutes, depending on the thickness and hardness of the agar blocks. Following exposure to the heat, the filter papers were removed and the resulting thin film of stained agar was mounted on a slide in Farrant's medium*. Stained preparations were examined within 4 days of preparation. The technique described was occasionally varied. With very soft agar, very thin 1 mm slices could be obtained directly from the agar plate with a small scalpel blade. Staining was sometimes allowed to proceed at 37°C in an incubator or at 45°C in an oven for periods ranging from 60 minutes up to 3 or 4 hours.

Preparation of stained coverslips.

**Coverslips stained were prepared as follows:- No. 1 microscope coverslips were cut into 10 mm. squares. They were cleaned in absolute alcohol and dried before staining solutions were applied to them. A large loopful (3mm) of 2 per cent. Toluidine blue was placed on the coverslip and to this a small loopful (approx. 2 mm) of 20 per cent. tartaric acid

* George Gurrs Ltd., England.

**Chance Proper Ltd., England.

was added, mixed and evenly spread and allowed to dry at room temperature. When the mixed solution had dried slightly the coverslip was gently rubbed with a filter paper to aid the crystallisation of the tartaric acid.

ELECTRON MICROSCOPY

Electron microscopic preparations were made of the following:

(i) glycine-induced L forms

(ii) L forms induced in liquid media

(iii) normal bacillary growth in hypertonic media

(iv) bacillary forms grown on isotonic medium.

The techniques used were modifications of the methods described by Kellenberger, Ryter and Schaud (1958).

L growth from liquid media, L growth from solid media suspended in liquid and normal bacilli harvested in normal saline were lightly centrifuged to sediment the cells and then held at 4-5^{o}C. These were then fixed in 3 per cent. gluteraldehyde made up in 3 per cent. buffered sodium chloride solution at 4^{o}C. (3 per cent. NaCl was prepared in 0.05M phosphate buffer). The pH of this fixative was adjusted to 7.2. The fixation of the cells was allowed for 10 minutes in an ice bath. Following this the cell suspensions were lightly centrifuged and fresh fixative

added which was cooled at 4°C for 4-6 hours. At the end of this period the fixative was replaced with phosphate buffer (pH 7.2) and the cells left overnight in the buffer. The cells were then treated in 1 per cent. osmium tetroxide (prepared also in phosphate buffer) for 2 hours in an ice bath. After the secondary fixation the cells were twice washed in buffer, sedimented and suspended in a small volume of agar gel. In the case of L colonies growing within the agar the colonies were excised with as little surrounding agar as possible and processed in the same way.

The cells were dehydrated and embedded as follows:

1. 30 per cent. alcohol(10 min.)
2. 50 per cent. alcohol(10 min.)
3. 70 per cent. alcohol(10 min.)
4. Absolute alcohol 2 changes (10 min. each)
5. Epoxy-propane 2 changes (15 min. each)
6. Araldite overnight at room temperature.
7. Fresh Araldite. The pelleted cells and colonies in agar were transferred and left up to 2 days at 37°C and then at 56°C to complete polymerization.

Sectioning was carried out in a Reichert ultra-microtome** and mounted on HR25 3 mm copper grids* and stained. The procedure adopted for staining is given below:

(a) Placed in saturated uranyl acetate in 50 per cent.

* Graticules Ltd., U.K.
**Reichert, Austria.

alcohol for 15 minutes.

(b) Washed twice in 50 per cent. alcohol followed by two washes with distilled water.

(c) In Reynold's (1963) lead citrate solution diluted with 0.01M NaOH for 5 minutes.

(d) Washed twice in 0.1 N NaOH and then in two changes of distilled water.

The stained sections were examined in an AEI 6B electron*microscope.

* AEI, U.K.

RESULTS

Possible differences were noted between field and laboratory strains (9S and 9R) of Salmonella gallinarum in their ability to transform under the influence of various L inducing agents and the results of the two groups are dealt with separately.

INDUCTION OF L FORMS IN LABORATORY STRAINS WITH PENICILLINS.

A systematic assessment was made of the cultural procedures and factors that influenced the production of L forms in strains 9S and 9R.

1. Ability of penicillin-treated liquid cultures to form L colonies on hypertonic solid media.

The bacillary forms of strain 9S were grown in peptone water (PW) and AJB medium at 37°C for 4-6 hours. To these cultures concentrated solutions of penicillin were added to give final concentrations of 4,000 to 40,000 units penicillin per ml. of culture. The treated broth cultures were further incubated at 37°C for 30-40 minutes before being plated out on hypertonic and isotonic media. The inoculated plates were incubated at 37°C. No L colonies developed on isotonic nutrient agar (NA) medium. The bacilli exposed to higher concentrations of penicillin produced bacillary colonies very much reduced in size on isotonic NA medium

(see plate 1), while cultures exposed to 4,000 units penicillin per ml. produced on NA medium a confluent type of growth which initially appeared as a thin film but with further incubation became thick and opaque. The small dwarf colonies which appeared on NA plates (i.e. bacillary colonies produced by cells exposed to high penicillin concentrations) grew in size with further incubation. On AJA medium and MC medium containing no penicillin but inoculated with penicillin-treated broth cultures (20,000 - 40,000 units penicillin/ml) the bacterial cells produced micro-colonies in 48 hours with incubation at 37°C. These colonies were extremely small, measuring less than 0.5 mm in diameter, and translucent. When stained by Gram's method these microcolonies consisted of masses of undifferentiated, pinkish staining material, thread-like whorls of tangled filaments and many grossly swollen bipolar stained 'rugby-ball' shaped forms. These microcolonies, when sub-cultured at 37°C on AJA medium containing no penicillin, produced L colonies after 7 days which were less than 1.0 mm in diameter and had the classical 'fried egg' appearance (see plate 2, 3, 4 & 5) and micro-L colonies (plates 6 & 7). Bacillary form in liquid media exposed to penicillin concentrations of 4,000 to 10,000 units/ml when subcultured on AJA and MC media containing no penicillin produced heteromorphic L colonies (see plates 8 & 9) with a classical morphology

and, in most instances, these colonies were covered with a confluent growth masking their identity. Confluent growth of this type occurred with prolonged incubation. The microscopic composition of this retrograde confluent heteromorphic L growth showed an admixture of swollen bacillary forms with large numbers of filamentous forms and small numbers of L elements. When stained by Gram's method none of the L elements withstood the staining procedure and appeared as a pinkish cloudy background material. The smears, however, showed numerous short filamentous forms and swollen bacillary forms (plate 11). The microscopic composition of the heteromorphic growth varied with the degree of exposure to penicillin (concentrations of penicillin in the broth cultures) and with the duration of incubation. Well developed heteromorphic L colonies with a classical morphology showed many large spherical bodies and few filamentous forms (see plate 10). Broth cultures exposed to more than 4,000 units of penicillin per ml of liquid medium were unable to multiply when subcultured on AJA or MC media in which penicillin had been incorporated at final concentrations of 80-12,500 units per ml of the medium. Only in one instance did L colonies form on MC media in which penicillin-treated broth cultures (10,000 units penicillin per ml of liquid media) were plated on MC media containing 200 units of

penicillin per ml. However, penicillin-treated PW or AJB cultures subcultured on AJA or MC media (in which penicillin was diffused from a trough/well cut out in the medium) produced L and heteromorphic L colonies. The production of these colonies was dependent on (a) the initial exposure dose of penicillin i.e. the amount of penicillin added to the broth cultures and (b) the amount of penicillin that was added to the troughs or wells; thus bacillary forms in liquid media exposed to 10,000 units of penicillin/ml of liquid medium, when plated out on AJA or MC medium in which 500 to 4000 units penicillin was allowed to diffuse, produced L colonies. Heteromorphic L colonies and a bacillary type of confluent growth were produced when either (a) the amount of penicillin that was added to the liquid cultures was lowered to 4000 units per ml of the liquid medium or (b) the amount of penicillin that was allowed to diffuse was lowered to 200 units. In these plates classical heteromorphic L colonies often coalesced or were overgrown by a confluent type of bacillary growth. No growth occurred when broth cultures exposed to high concentrations of penicillin (40,000 units penicillin per ml of liquid media) were subcultured on AJA medium in which high concentrations of penicillin were allowed to diffuse (40,000 units penicillin instilled in the well/trough). Treated PW cultures were equally as effective as

treated AJB cultures in producing L and heteromorphic L colonies under appropriate conditions.

2. Ability of untreated broth cultures to produce L transformation directly on hypertonic solid medium containing penicillin at 37°C.

Four- to 6-hour PW and AJB cultures of 9S and 9R incubated at 37°C were plated out on hypertonic AJA and MC media containing varying concentrations of penicillin and also on similar media without penicillin. Untreated liquid cultures, plated out on AJA and MC media not containing penicillin, produced within 24 hours' incubation a confluent bacillary growth which, on further incubation, became thick and opaque. Microscopically only bacillary forms were present in these cultures. The bacillary forms obtained on hypertonic media were short and swollen compared with normal rod forms obtained on NA medium (see plate 12). Untreated liquid cultures subcultured on AJA and MC media in which penicillin was incorporated to give final concentrations ranging from 80-400 units/ml produced patchy granular and heteromorphic L type growth. On AJA and MC plates containing 80 units/ml penicillin, there developed a confluent growth consisting predominantly of normal and swollen rods and distorted and swollen filamentous forms. There were also small numbers of large round pale-staining bodies and large

and medium-sized spherical elements. The microscopic appearance of this growth was not unlike the revertant type growth seen in plate 13, except that small and tiny spherical bodies were absent. When the penicillin concentration in the induction medium was increased to 200 units/ml growth of L forms and bacillary elements was inhibited but occasionally at this concentration a patchy granular L growth (resembling those shown in plate 14) consisting predominantly of L elements developed, but generally died out; likewise, in induction media containing 400 units/ml penicillin only rarely did a patchy granular L type growth appear which showed no further colonial development. Untreated liquid cultures plated out on AJA and MC media in which penicillin was allowed to diffuse produced growth in which the degree of transformation was determined by the amount of penicillin that was diffused; thus when 200 units of penicillin was diffused in these solid hypertonic media the growth produced was bacillary in nature consisting predominantly of swollen bacilli and filamentous forms. When the amount of penicillin that was diffused was increased to 400 units a confluent growth was produced which, when examined microscopically between the 5th and 6th days of incubation was seen to consist of filaments showing varying degrees of thickening together with a few spherical elements

In these plates there was usually a wide zone of no growth around the penicillin wells or troughs. In plates containing 500 units penicillin diffused from well/trough heteromorphic confluent growth developed. Some of these colonies grew into the agar and could be identified grossly as dark spots studding the agar. Microscopically they were comprised predominantly of filamentous forms exhibiting various degrees of swelling and thickening along their length and small numbers of L elements interspersed among the filamentous transitional forms (see plates 15,16,17). In plates of AJA medium in which 40,000 units of penicillin had diffused the heteromorphic L growth, which often appeared as coalesced classical type colonies, consisted predominantly of transitional elements (filamentous forms with gross enlargements and thickenings) and large numbers of spherical bodies. The spherical bodies were more in number than in the heteromorphic growth produced at lower penicillin concentrations. The microscopic composition of these was very similar to the well developed classical heteromorphic L colonies (see plate 10).

3. Effect of added divalent cations:

No marked stimulatory effects were obtained by the addition of calcium chloride to the AJA medium (MC medium). Penicillin-treated cells exhibited the

same degree of L transformation and growth characterstics under similar conditions of cultivation, the only exception to this being the production of L colonies on MC medium containing 200 units/ml of penicillin from cells exposed to 10,000 units penicillin per ml of the liquid culture, whereas on AJA medium no growth was produced. M medium containing approximately the same amount of calcium chloride as in MC medium but more magnesium sulphate than in AJA and MC media, was found to have a stimulatory effect on transformation of penicillin-exposed cells to transitional and L elements. AJA medium inoculated with penicillin-treated liquid cultures (4,000 units penicillin per ml of the liquid medium) produced heteromorphic L type growth in which the transitional elements were numerically more than the L elements, while under identical cultural conditions on M medium, the heteromorphic growth produced proportionately more L elements, and the qualitative changes were more marked. Gram's staining of these colonies showed only a few grossly swollen and serpentine transitional forms against a large mass of pinkish nondescript background indicating that most of the L elements had been damaged by the process of staining and thus suggesting the extent of cell transformation.

The yield of these colonies was, however, considerably poorer on M medium than on

AJA medium. The increase of magnesium sulphate in AJA medium from 0.2 to 0.3 per cent. had no noticeable stimulatory effect. It thus appeared that both the divalent cations were necessary for cell transformation, and that the beneficical effects were more apparent when the magnesium sulphate content in the medium was at least 0.5 per cent. In order to obtain a better yield of L elements in the presence of higher concentrations of divalent cations a medium was devised which contained the same amounts of calcium chloride and magnesium sulphate as in the M medium and some of the medium components from AJA medium. This medium (MAJA) did not produce the desired effects as far as L transformation was concerned. Penicillin-treated broth cultures (10,000 units penicillin per ml of liquid medium) subcultured on MAJA medium which had 500 units of penicillin diffused from a well, did not produce L growth, whereas the same cultures plated out on AJA medium containing the same amount of penicillin produced classical L colonies. However, on MAJA plates containing no penicillin, the same penicillin-treated cells produced a luxuriant confluent bacillary growth (consisting mostly of rods and large numbers of filaments, with very few L elements) while on similar AJA plates devoid of penicillin the bacillary type of growth was not evident even on the 7th day of incubation.

4. Effect of addition of penicillin to broth cultures at different growth phases.

These experiments were carried out to determine whether bacillary forms in a particular stage of physiological activity were particularly susceptible to L transformation. AJA and M media were used in these experiments. PW media were inoculated with 10^7 cells and incubated at 37°C (see page 188). Penicillin (10,000 units/ml) was added to 3 hour 30 minutes, 4 hour 15 minutes and 5 hours 25 minutes and overnight (i.e. >12 hour) incubated cultures and allowed to act for 30 minutes. These penicillin-treated cultures were subcultured on AJA and M media containing no penicillin. No marked differences were noted in the ability of cells to transform to L elements at any particular phase of bacterial growth, although pre-exponential cultures (3 hour 40 minute incubated) treated with penicillin produced the least bacillary forms on solid media.

5. Effects of penicillin exposure time in relation to L transformation.

The bacillary forms grown in AJB medium were incubated at 37°C for approximately 6 hours following which penicillin was added to give a final concentration of 40,000 units penicillin per ml of the medium. The broth cultures containing penicillin were further

incubated at 37°C and at intervals of 0, 15, 30 and 60 minutes following the addition of penicillin, they were subcultured on AJA and M media. L elements were produced on all the plates inoculated with treated broth cultures but the proportion of L elements to the transitional and bacillary forms varied. L elements were numerically more in plates inoculated with broth cultures exposed to the action of penicillin for 60 minutes. A similar experiment was carried out using a 23-hour incubated culture of strain 9S to which penicillin was added and subcultures were made on M medium at varying intervals following the addition of penicillin. The results of this experiment also indicated that a greater proportion of the bacillary forms transformed into L elements in plates that had been exposed to the action of penicillin for 60 minutes.

6. Effect of temperature of incubation on L induction.

Temperature of incubation proved to be not only a very critical factor in the development of L forms but also to have an effect on the survival of bacillary forms exposed to the action of penicillin. An eighteen-hour PW culture (incubated at 37°C) of strain 9S containing 2.8×10^7 cells/ml of the liquid medium was inoculated on AJA medium containing 100 units penicillin/ml of the induction medium. The inoculated plates were incubated at 30 and 37°C. Following 24 hours' incubation, the

plates that had been incubated at 30°C showed a patchy granular L type growth (see plate 14). This growth, when examined microscopically, showed predominantly L elements (large, round pale-staining bodies and large and medium sized spherical elements) with very few transitional forms. The large round pale-staining bodies were generally 20-50mμ in diameter and stained less intensely than the spherical elements which were 10-20mμ (large sized) or 5-10mμ (medium sized) in diameter; the few transitional forms seen consisted of long rods with bulbous swellings, short filaments with saccular enlargements and giant 'sausage-shaped' forms. The plates incubated at 37°C following 24 hours' incubation showed no growth. The 30°C plates showed more areas of patchy granular L type growth after 48 hours' incubation, while the corresponding plates incubated at 37°C showed no growth up to the 13th day of incubation. With strain 9R, cultivated under identical cultural conditions, a similar effect was apparent, the only difference being that the plates incubated at 37°C showed a small amount of patchy granular L type growth which remained poorly developed and died off with further incubation. The effects of the temperature of incubation on L transformation was also studied on media containing 10 per cent. inactivated horse serum in the presence of penicillin (100 units per ml of the induction media). PW cultures of strain 9S plated out on AJA medium

containing 10 per cent. inactivated horse serum and 100 units penicillin/ml showed a patchy granular L type growth in both the 30°C and 37°C. plates following 24 hours of incubation. After 3 days' incubation plates at 30°C showed a moderate to heavy patchy granular L type growth and also the development of heteromorphic L type colonies (see plate 18) whereas in the 37°C plates there was very little colonial development. By the seventh day of incubation both 30°C and 37°C plates had classical heteromorphic L colonies as well as well developed patchy granular L type growth. Microscopical examination of the classical heteromorphic L growth on the 37°C plates showed that these colonies were in the process of reversion. In the plates inoculated with strain 9R the 37°C plates showed a faint patchy granular L type growth while the 30°C plates showed a more profuse patchy granular L type growth. After 3 days'incubation the differences in the 30°C and 37°C plates were more marked. In the 30°C plates large numbers of classical heteromorphic L type colonies appeared whereas in the 37°C plates there was only an occasional classical heteromorphic L colony and patchy granular L type growth which was already showing signs of reversion.

7. Effects of serum in induction media

The presence of serum in induction media appeared to

favour the development of L elements. To examine the growth promoting properties of serum, liquid cultures of the bacillary forms were subcultured on penicillin-containing AJA medium in which 10 per cent. inactivated horse serum was incorporated and on similar media not containing serum. The beneficial effects of serum were most marked with increased concentrations of penicillin in the induction medium. Liquid cultures of the bacillary forms of Strain 9S (2.8×10^7 cells per ml) were seeded on AJA medium containing 100 units penicillin/ml and 10 per cent. inactivated horse serum, and on similar medium containing no serum. After 24 hours' incubation at 30°C, in both types of media patchy granular L type growth developed, the growth being more profuse in plates containing serum. In plates incubated at 37°C the effect of serum were more noticeable in that while the AJA medium containing no serum did not support L type growth, the medium in which serum (10 per cent.) was incorporated, produced a faintly developed patchy granular L type growth within 48 hours of incubation. After 7 days' incubation at 37°C the medium containing serum showed a number of classical heteromorphic L colonies, which were in the process of reversion. When the penicillin concentration in the induction medium was increased, serum was found to be critical for the development of altered variants; thus AJA medium containing 150 units penicillin per ml. of

the induction medium (not containing serum) inoculated with 5.5 x 10^8 bacilli/ml of strain 9S failed to produce any form of growth with up to 16 days of incubation at 30°C, while on a similar medium containing serum, a patchy granular L type growth was apparent on the second day of incubation. This growth persisted up to the 12th day of incubation and in addition classical L type colonies were produced. When the penicillin concentration was increased to 400 units/ml in the induction media no growth was produced in medium in which serum had been excluded, whereas in AJA medium with 10 per cent. serum a very faint patchy granular growth developed within 24 hours' incubation at 30°C which, by the 48th hour of incubation, had become more extensive. No further colony development took place on these plates up to the 15th day of incubation. When they were re-examined on the 19th day of incubation signs of reversion were evident. The film of confluent growth that had developed consisted predominantly of rods with a few L elements and occasional transitional forms. Parallel experiments were carried out with strain 9R. As in strain 9S, 9R behaved in a similar fashion in that the growth-promoting effect of serum on L forms was noticeable in induction media containing high concentrations of penicillin.

Increasing the serum content of the induction medium from 10 to 20 per cent. had the effect of bringing about

increased conversion of the bacillary forms to L elements. Four-and-a-half-hour incubated concentrated PW cultures (see page 197) of strains S and 9R were plated out on AJA medium containing 200 units penicillin/ml and 20 per cent. inactivated horse serum and on similar medium containing no serum (as the addition of such large volumes of serum softened the consistency of the medium, the medium containing no serum was 'softened' to the same extent by the addition of sterile water to the molten agar). The inoculated plates were incubated anaerobically at 37°C. When the plates were examined after 4 days' incubation, AJA medium in which 20 per cent. inactivated serum had been incorporated showed extensive and well developed patchy granular L type growth as well as large classical L type colonies on the surface of the agar and a number of large globular atypical colonies within the depths of the agar (see plate 19). These atypical colonies consisted predominantly of L elements and a few transitional elements; rod forms were completely absent. In similar plates containing no added serum and seeded with the same inoculum, a confluent revertant type of growth was produced which microscopically consisted mostly of bacillary and transitional elements. No well demarcated L colonies were produced either on the surface of within the agar.

A further effect of serum was to hide the superiority

of M medium over the AJA medium in bringing about L transformation. Salmonella gallinarum strains 9S and 9R were grown in PW at 37°C for 22 hours. PW cultures (1.35 x 10^8 cells/ml) of these strains were subcultured on M and AJA media containing 10 per cent. inactivated horse serum and 40 units penicillin/ml. The inoculated plates were incubated at 37°C. The addition of serum to M medium caused clouding of the medium which was particularly noticeable after incubation so that the gross morphology of colonies developing on these plates could not be observed properly. Inoculated M plates showed areas of confluent growth and isolated small colonies after 3 days incubation. These colonies, when stained and examined by both Gram's and Dienes' methods were found to consist of medium sized rods and to a much lesser extent of filaments many of which had gross swellings and enlargements. A few spherical elements were also present in this type of growth. On the AJA medium containing similar amounts of penicillin and serum and under identical cultural conditions, heteromorphic L type growth as well as bacillary growths were produced. With strain 9R no transformation occurred on M medium containing 10 per cent. inactivated serum and 40 units/ml penicillin while in the corresponding AJA medium a small number of L elements was seen in the confluent growth.

8. Effects of inoculum size.

Inocula low in viable cell count generally produced poor and irregular growth on induction media containing more than 100 units of penicillin/ml or more than 1.5 per cent. glycine or in media containing 1.5 per cent. glycine and 50 units penicillin/ml. The effect of inoculum size in relation to growth, yield and development of cell wall defective variants are well illustrated in the following experiments. Salm.gallinarum strain 9S was grown in PW for 21 hours at 37°C. The cells were sedimented by centrifugation, washed twice in normal saline and reconstituted in normal saline to give cell concentrations of 5.5×10^8, 5.5×10^7, 5.5×10^6 and 5.5×10^4 bacilli/ml. A volume of 0.1 ml. of each dilution was plated out on AJA medium containing 10 per cent. inactivated horse serum and 150 units penicillin/ml of the induction medium; thus, plate A seeded with 0.1 ml of 5.5×10^8 cells received approximately 5.5×10^7 cells and plates B, C and D were seeded with 5.5×10^6, 5.5×10^5 and 5.5×10^4 bacilli respectively. The inoculated plates were incubated at 30°C. After 48 hours' incubation a patchy granular L type growth developed on plates A and B. These findings remained unchanged until the 9th day of incubation. By the 12th day of incubation in B plate, the patchy granular L growth had become more extensive and covered more areas

of the plate while in plate A large classical L type colonies were produced (see plate 20). No bacillary or L growth occurred in plates C and D over the 16 days of incubation. Parallel experiments were carried out with strain 9R using similar inoculum sizes with identical media and cultural conditions. As with strain 9S, plate A inoculated with strain 9R showed a patchy granular L type growth (the growth being less extensive) within 48 hours of incubation at 30°C, while in plates B, C and D no growth occurred. The patchy granular L growth in plate A was well developed by the 3rd day of incubation and by the 12th day approximately 25 large classical L type colonies were seen in these plates while in plates B, C and D, no growth was visible. The effects of inoculum size in glycine-containing and in combined glycine-penicillin media, were studied by arbitrarily diluting concentrated PW cultures so that no estimations of the cell content of the inoculum were made. The results of these observations on glycine and glycine-penicillin induction media also suggested that low inoculum sizes gave only poor growth on such media.

9. Effect of pH on L induction

The effect of hydrogen ion concentration of the induction medium (AJA) on L colony production was studied at three pH values viz. 6.7, 7.2 and 8.1. The induction media contained 100 units/ml penicillin

and were seeded with 18-20 hr. PW cultures of strains 9S and 9R. Inoculated plates were incubated at 30°C. After 3 days' incubation a moderately well developed patchy granular L type growth appeared on the AJA media in which the pH had been adjusted to 7.2, the growth being abundant. The patchy granular L type growth on medium at pH 7.2 at this stage of development consisted almost entirely of L elements (predominantly large round, pale-staining bodies and large spherical elements but to a lesser extent medium and small spherical elements) together with a small number of transitional elements (similar to those shown in plate 21) and distorted L elements. On the medium at pH 6.7 the growth after 3 days was of a heteromorphic confluent L type and when stained both by Gram's and Dienes' methods, showed a large proportion of transitional forms with moderate numbers of spherical bodies (large, medium and small) and a few rod forms and large round, pale-staining bodies. By the 7th day of incubation a qualitatively better type of confluent heteromorphic L type growth was obtained (media with pH 6.7) in that there were proportionately more L elements. Bacillary forms were present only in very small numbers and consisted of extremely long rods (?short filaments). Classical heteromorphic L colonies developed on media with a marked alkaline reaction (pH 8.1) after 3 days' incubation at 30°C. These 3-day-old colonies consisted

mainly of transitional forms showing gross enlargements and swellings and large collections of L elements with a preponderance of the small spherical elements. These colonies, when examined on the 7th day of incubation, consisted of medium sized rods and coccal forms with small numbers of L elements and transitional forms. This suggested reversion changes. The growth of strain 9R on the same media with varying pH values was investigated using identical cultural conditions. On the medium with pH 6.7 a thick opaque and moist growth developed on the 3rd day of incubation; this growth, when stained by Gram's and Dienes' staining techniques, consisted predominantly of L elements (large and medium sized spherical bodies and many large pale-staining round bodies and a few small and tiny spherical bodies). The filamentous transitional forms were also present in large numbers although numerically less than the L elements but with an almost complete absence of rod forms. By the 7th day of incubation the filamentous forms were more abundant than the L elements and many bacillary forms were present indicating a process of reversion unlike strain 9S.

On the medium with pH 7.2 inoculated with strain 9R the 3-day growth in gross appearance was not unlike that present in the pH 6.7 medium; microscopically this growth was seen to consist of large numbers of L elements and filamentous transitional forms, the latter

being the predominant forms. A few long and very occasional medium sized rod forms were present. A large number of coarse granules were also present which stained dark blue by Dienes' stain. This heteromorphic L growth when examined on the 7th day of incubation showed more filamentous forms and marginally more rod forms than on the 3rd day of growth, indicative of reversion. On media with pH 8.1 strain 9R gave a heavy confluent moist and mucoid type of growth after 3 days' incubation at 30°C. Stained preparations of this growth (heteromorphic L growth) showed predominantly filamentous forms and fewer L elements with many transforming as well as normal rod forms. The growth when examined microscopically on the 7th day of incubation was seen to consist mainly of long and medium sized rods, a few to moderate numbers of transitional elements (mainly filaments) and a very small number of L elements (only a few large round pale-staining bodies and large, medium and small sized spherical bodies present (see plate 13). This revertant type growth had more normal bacillary elements and less L and transitional elements than had strain 9S grown on medium of the same pH.

10. Miscellaneous factors affecting L production.

The omission of glycerol was without noticeable effect on L form induction; AJA medium in which glucose

was autoclaved in the presence of other medium components was found to be inhibitory for the production of L elements, particularly when freshly prepared medium was used.

11. The efficacy after storage of AJA medium with added penicillin in inducing L transformation.

Considerable loss of penicillin activity was noted when AJA medium containing penicillin was stored at room temperature (14-18°C). The efficacy of L transformation in AJA medium in which the penicillin (200 units/ml) and inactivated horse serum (10 per cent.) had been incorporated a few hours prior to use was compared with that in AJA medium in which the addition of penicillin and horse serum has been made 10 days previously and the prepared plates stored at 14-18°C. AJA medium containing serum but no penicillin, both freshly prepared and stored for 10 days, served as controls. The plates were inoculated with 19-hour PW cultures of strains 9S and 9R containing 8×10^8 bacilli/ml and were incubated at 30°C. After 24 hours' incubation both control media showed a thick confluent bacillary growth covering the surface of the medium. Neither of the control plates showed any growth of colonies into the medium and microscopically it was found to consist entirely of bacillary elements. The stored medium containing penicillin produced after 24 hours' incubation a thin

film of confluent growth which became more profuse after 48 hours and was found on examination under the low power (x5) objective of the microscope to be studded with darker, well circumscribed small areas of growth. The confluent growth appeared microscopically to consist mainly of medium sized and long rods, with moderate numbers of short filaments and to a lesser extent long filamentous forms showing uniform thickenings and occasional spherical bodies; the growth at 72 hours consisted of the same elements except that the spherical bodies had disappeared. At both 48 and 72 hours removal of the surface growth revealed well circumscribed areas of growth into the agar corresponding to the dark areas already described (see plate 23). Microscopical examination of preparations from those areas showed the growth to consist predominantly of L elements of varying sizes (plate 24).

In AJA medium to which freshly prepared solutions of penicillin has been added, examination at 24 hours showed a hardly visible patchy granular L type growth which by the 3rd day of incubation became more extensive and opaque and when stained by Dienes' method was found to be completely devoid of bacillary elements, consisting mainly of L elements of varying sizes with very few transitional forms (see plate 21)
On the 4th day of incubation a single small (less than 0.5 mm) colony was present in the depths of the agar.

By the 10th day a large number of L and heteromorphic L colonies had developed. The L colonies were made up of large, medium, small and tiny spherical elements; large numbers of granules, a few large round pale-staining bodies and few to moderate numbers of transitional elements (see plates 25 & 26) were also observed.

12. Induction of L forms on defined synthetic and semi-synthetic media.

MKS medium and a semi-synthetic medium (MKS plus 10 per cent. horse serum) were used in these studies. Twenty-four hour PW cultures of strains 9S and 9R were plated out on MKS and MKS-serum media containing 100 and 200 units penicillin as well as on each medium without penicillin. Plates were incubated at 30°C and examined at the 3rd, 9th, 11th and 15th days of incubation. MKS plates containing 100 and 200 units penicillin failed to support any growth. On MKS-serum medium containing 200 units of penicillin no growth occurred while on plates containing 100 units a faint confluent growth consisting predominantly of filamentous and bacillary forms was seen in a few areas of the plate on the 9th day of incubation. On MKS and MKS-serum media with no penicillin the bacillary growth was scanty.

13. Induction of L forms on K media.

Overnight concentrated PW cultures of strains 9S and 9R were plated out on K medium containing 100 and

250 units/ml penicillin, and on K medium containing no penicillin. After inoculation plates were incubated at 30°C. Plates containing 250 units/ml penicillin failed to support any growth but on plates containing 100 units/ml of penicillin a faint growth containing a mixture of bacillary and filamentous forms was seen. Control plates of K medium without penicillin supported poor bacillary growth. This medium was found to produce precipitation of the salts present in the media and was unsatisfactory for observation of colonial morphology and staining by Dienes' technique.

14. Induction of L colonies by the pour plate method.

Induction of L forms by the pour plate method was carried out on AJA medium containing 10 per cent. horse serum with 200 units penicillin/ml of the medium. Control media were also included (viz. AJA medium with and without serum but containing no penicillin). The inoculum consisted of a 20-hour PW culture of strain 9S incubated at 37°C. Penicillin and serum were added to molten AJA medium held at 45-50°C to produce the required concentrations of these two substances. Between 0.1 to 0.2 ml of the inoculum was placed in a 50 mm plastic petri dish and the molten induction medium added. To ensure an even distribution of the inoculum the plates were gently rocked and then left at room temperature for 1-2 hours to solidify before they were

incubated. All inoculated plates were incubated at 30°C.

AJA medium containing 200 units penicillin but without serum failed to support bacillary or L growth over the 16 days of incubation. In the AJA medium containing serum no growth was present up to the 9th day of incubation but when the plates were re-examined on the 13th day a few compact round colonies were found growing within the depths of the agar and a thin film of bacillary growth had developed on the surface of the medium. The surface growth consisted of swollen bacillary forms, transitional filamentous forms showing varying degrees of swellings and thickenings and large numbers of large and medium sized spherical elements with a few large round pale-staining bodies. This growth appeared to be a confluent heteromorphic L type growth in the early stages of reversion. On the other hand, the colonies growing within the depths of the agar were L colonies which, when stained by Dienes' method, were composed almost entirely of L elements (large, medium, small and tiny spherical elements) with very few transitional forms. In medium containing no penicillin a confluent surface bacillary growth developed within 24 hours and after 48 hours bacillary growth was also present within the depths of the agar. Smears of the growth on these plates showed only bacillary elements. Parallel experiments carried out with strain 9R produced

slightly different results in that heteromorphic growth (both confluent and discrete colonies) developed on AJA medium containing 200 units penicillin (but not containing serum). The heteromorphic colonies appeared on the 3rd day of incubation and by the 9th day more colonies had appeared. The heteromorphic L growth on these plates was less well transformed in that there were large numbers of rod and filamentous transitional forms. A surface growth appeared on these plates on the 13th day of incubation and this, when examined microscopically, consisted predominantly of rods with moderate numbers of filaments and to a lesser extent L elements. On AJA medium containing serum with 200 units/ml penicillin L colonies appeared within the depths of the agar by the 7th day of incubation. This growth, stained by Dienes' technique, consisted of L elements and less than 10 per cent. transitional elements. More transitional elements were present in these L colonies than in those produced under identical conditions by strain 9S.

15. Controls

In all the foregoing experiments appropriate cnntrols were included. In no instance were L elements produced with strains 9S and 9R by untreated (i.e. without added penicillin) PW and AJB cultures on any of the isotonic or hypertonic medium (not containing penicillin). Likewise, serum per se did not bring about any transformative changes in these two strains; no gross

differences were noticed in the cell morphology of bacillary cultures grown on isotonic medium at 30°C and 37°C., but on hypertonic medium the normal rod forms (see plate 12) became short and turgid and assumed a swollen coccobacillary form at both temperatures (see plate 27). Likewise the bacillary forms were more turgid and rounded on media constaining serum than those not containing serum. There was also a greater tendency for the production of filaments on AJA medium containing serum particularly with strain 9S.

PROPAGATION OF L FORMS (LABORATORY STRAINS 9S AND 9R)

1. Propagation of L forms at 37°C on AJA and M media not containing serum.

L colonies when subcultured on AJA medium and M medium at 37°C produced a variety of growth forms depending largely on (a) the penicillin concentrations in the propagating medium and (b) the type of L growth that was subcultured i.e. the microscopical composition of the L growth. Well developed classical L type colonies containing minimal numbers of transitional forms when subcultured on AJA medium and M medium diffusion plates (penicillin diffused from trough/well) containing 500 to 40,000 units penicillin produced heteromorphic L colonies with a slightly altered morphology (see plate 28). These colonies had a large dark central core and a narrow lighter periphery and were comprised essentially of the same elements found in classical L colonies except that there were proportionally more transitional forms and in addition a small number of bacillary forms were present. These heteromorphic L colonies appeared between the 5th and 9th days of incubation, and when they were subcultured on AJA penicillin diffusion plates (500 units penicillin in agar well) produced two types of growth namely (a) a similar type of heteromorphic L colony which appeared between 4th and 5th days of incubation close to the penicillin source

and (b) a confluent type of growth which appeared between the 3rd and 5th days of incubation further away from the penicillin source. This confluent growth consisted predominantly of transitional filamentous forms and large numbers of bacillary forms. A few L elements were interspersed among the transitional and bacillary forms. Essentially the growth was of a revertant type very similar to that shown in plate 13 except that small spherical elements were generally absent. In a number of instances when the discrete heteromorphic L colonies were subcultured on AJA penicillin diffusion plates (500 units penicillin diffused from a central well) there was growth of L colonies within the depths of the medium. These colonies could be more easily seen when the overlying surface growth was removed (either by blotting it out with a filter paper or washing the confluent growth off the surface) and appeared as dense compact structures growing into the agar (see plate 23). The dense compact colonies were generally present only under the confluent growth found nearer the penicillin wells. L colonies and heteromorphic L colonies subcultured on AJA medium in which penicillin had been incorporated to give a uniform concentration of 2000-4000 units per ml. of the medium failed to grow. L colonies and heteromorphic L colonies subcultured on AJA medium and M medium containing no penicillin rapidly reverted to the

bacillary state within 48 hours, but occasionally L colonies were produced which were overgrown with the revertant type of bacillary growth.

The microcolonies that were produced on AJA medium and M medium from penicillin-treated broth cultures and in penicillin diffusion plates inoculated with untreated PW cultures constituted a type of heteromorphic L growth. These colonies differed from other heteromorphic and classical L colonies in being more translucent and of smaller size. When these microcolonies were subcultured within 24 hours of their appearance on to penicillin diffusion media (penicillin diffused from trough/well containing 4000 units they produced classical L colonies, but on diffusion plates containing lower concentrations of penicillin (500 units in well) they produced heteromorphic L colonies. Microcolonies of this type subcultured on M medium containing no penicillin were slower to revert than those produced and propagated on AJA medium.

2. Propagation of L forms at 37°C on AJA and M media containing 10 per cent. inactivated horse serum.

Microcolonies produced on M medium and subcultured on a similar medium containing 10 per cent. horse serum and 100 units/ml penicillin produced larger classical heteromorphic L colonies. When these microcolonies were subcultured on AJA serum medium containing 200

and 300 units penicillin diffused from an agar well, they produced L colonies, heteromorphic L colonies and confluent bacillary growth. The bacillary growth appeared to become more profuse with prolonged incubation. Classical L colonies produced on AJA medium and then subcultured to AJA medium containing 10 per cent. horse serum and penicillin (100, 200 and 300 units penicillin per ml of the propagating medium) produced L colonies, patchy granular L type growth and heteromorphic L growth, and generally after the 4th day of incubation these colonies reverted. When the reverting L colonies (see plate 29) were subcultured on serum AJA medium containing similar concentrations of penicillin the growth became more confluent and was more bacillary in nature.

3. Effects of temperature of incubation of the propagation of L forms in media with and without added serum.

A better growth of L forms was obtained in subcultures on AJA medium containing 10 per cent. horse serum incubated at 30°C than on media containing no added serum or on serum AJA medium incubated at 37°C. Incubation at 37°C favoured reversion and the development of bacillary elements. Subcultures were made from large classical L type colonies grown for 3 days on AJA medium containing serum on to AJA medium without serum but with 100 units/ml of penicillin and on to AJA medium with 10

per cent. horse serum and incubated at 30°C and 37°C. After 4 days a faint patchy granular L type of growth was present on the AJA medium containing penicillin but no serum, incubated at 37°C. Further examination on the 6th and 10th days of incubation revealed no further colony development, and by the 16th day the growth had died off. Cultures on the same medium incubated at 30°C showed large heteromorphic L type colonies (similar to those shown in plate 18 and 20) many of which were coalescing by the 4th day of incubation. Microscopically these colonies were characterised by the large numbers of filamentous transitional forms and considerably fewer L elements which were of the large and medium variaties with a few small spherical bodies also present (like those in plate 15, 16 & 17). This type of colony constituted a poorly transformed heteromorphic L growth. The cultures on AJA medium containing serum incubated at 37°C for 48 hours showed a confluent reverting heteromorphic L type growth studded with dark circular spots. These circular areas represented L colonies growing within the agar. The confluent growth consisted predominantly of bacillary elements, filamentous transitional forms and a few L elements. The L colonies growing into the agar consisted predominantly of L elements with moderate number of transitional forms. The corresponding plates incubated at 30°C showed numerous well separated classical L colonies.

Serial passage of L, heteromorphic L and reverting L colonies on penicillin-containing media.

Serial subcultures of L, heteromorphic L and reverting L colonies were carried out in order to (a) produce a more homogeneous L type growth from heteromorphic L colonies, (b) retransform reverting L colonies to heteromorphic or L type colonies and (c) study the changes produced in cell and colony morphology in L and heteromorphic L type growth as a result of prolonged subculture in media containing penicillin. The general outline of the serial subcultures and the growth types obtained are illustrated schematically in the next pages in which the following abbreviations are used:-

ANO_2 = Anaerobic incubation

PW9S = Peptone water cultures of strain 9S

PW9R = Peptone water cultures of strain 9R

AJA = Alexander-Jackson medium

NAJA = Modification of the above medium (see Materials and Methods)

u-pen = units penicillin/ml

HL = Heteromorphic L colonies

37^{o})
) denote temperature of incubation (degrees
30^{o}) Centigrade)

S10)
) denote percentage horse serum (inactivated)
S20) incorporated into the media.

↓ = subculture

\+ = additional derivative from same culture.

The last 3 arabic numerals following medium notation indicate the plate number (but does not indicate the number of subcultures) thus AJA-S10/011 denotes AJA plate No. 11 containing 10 per cent. inactivated horse serum while AJA/114 denotes AJA plate No. 114 containing no serum. The letter R before the last three numbers denotes Rough Strain (9R).

Unless otherwise stated penicillin has been incorporated into the medium to give a uniform concentration

TABLE 2

Serial subculture of L, HL and revertant colonies of 9S

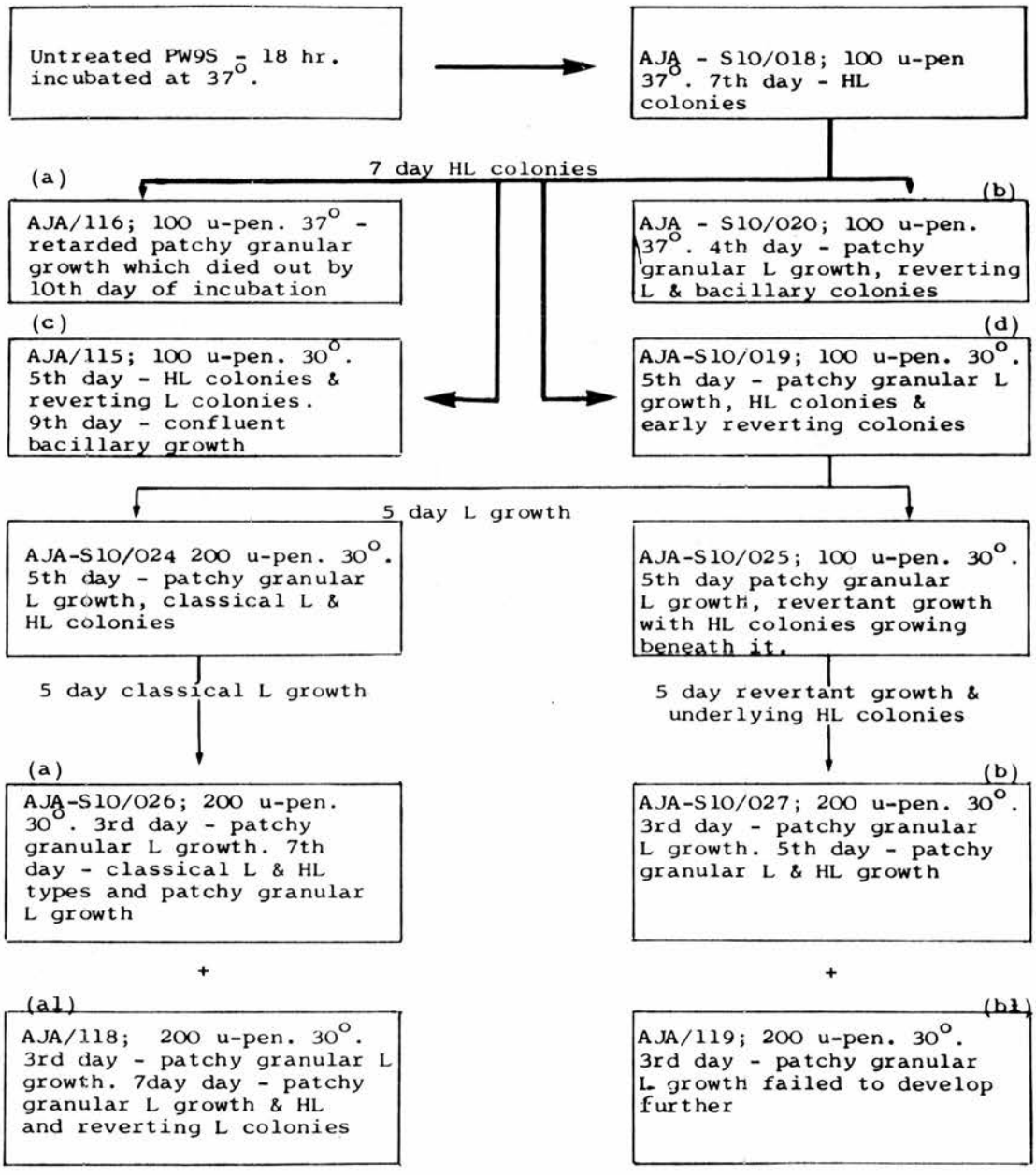
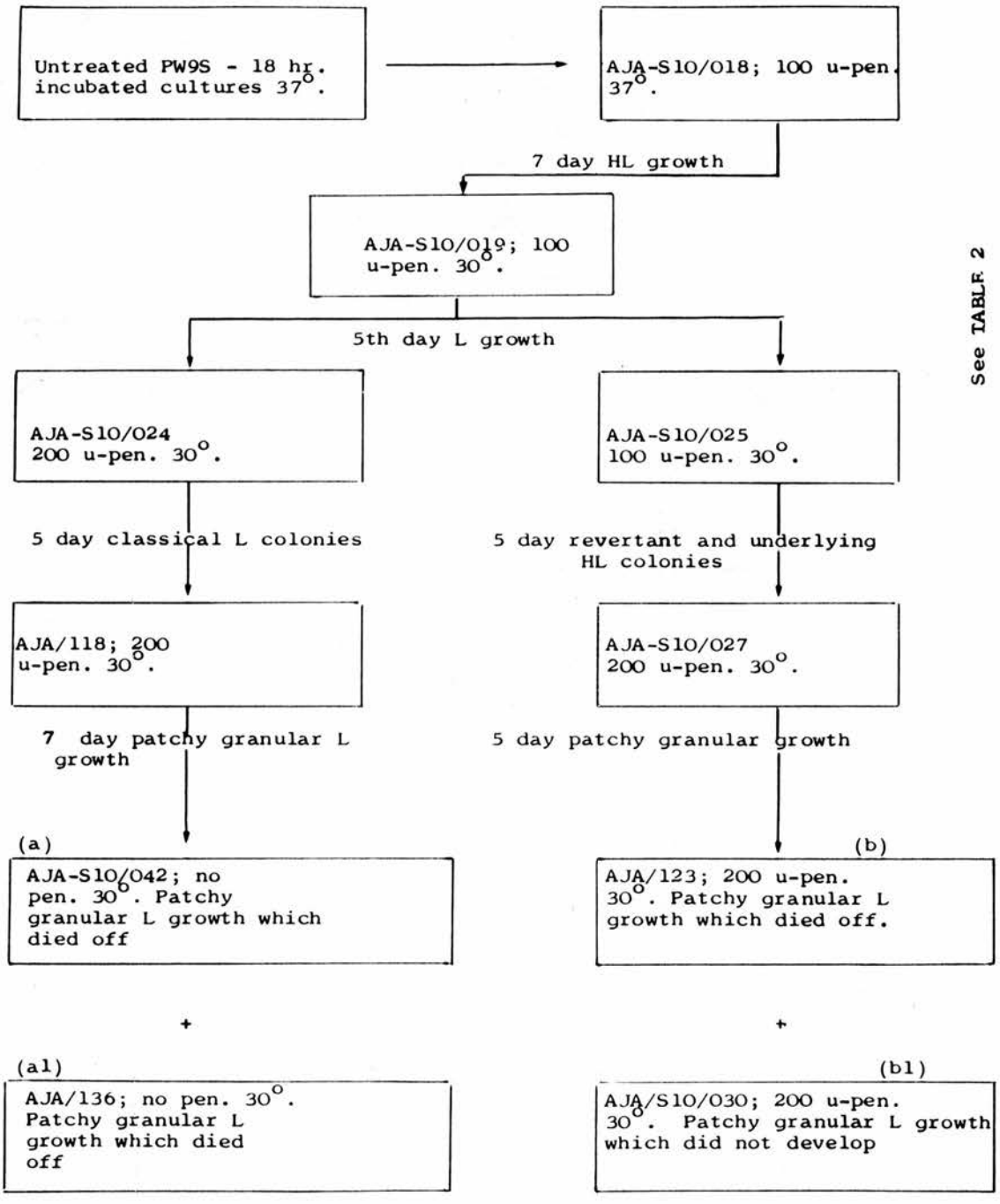


TABLE 3

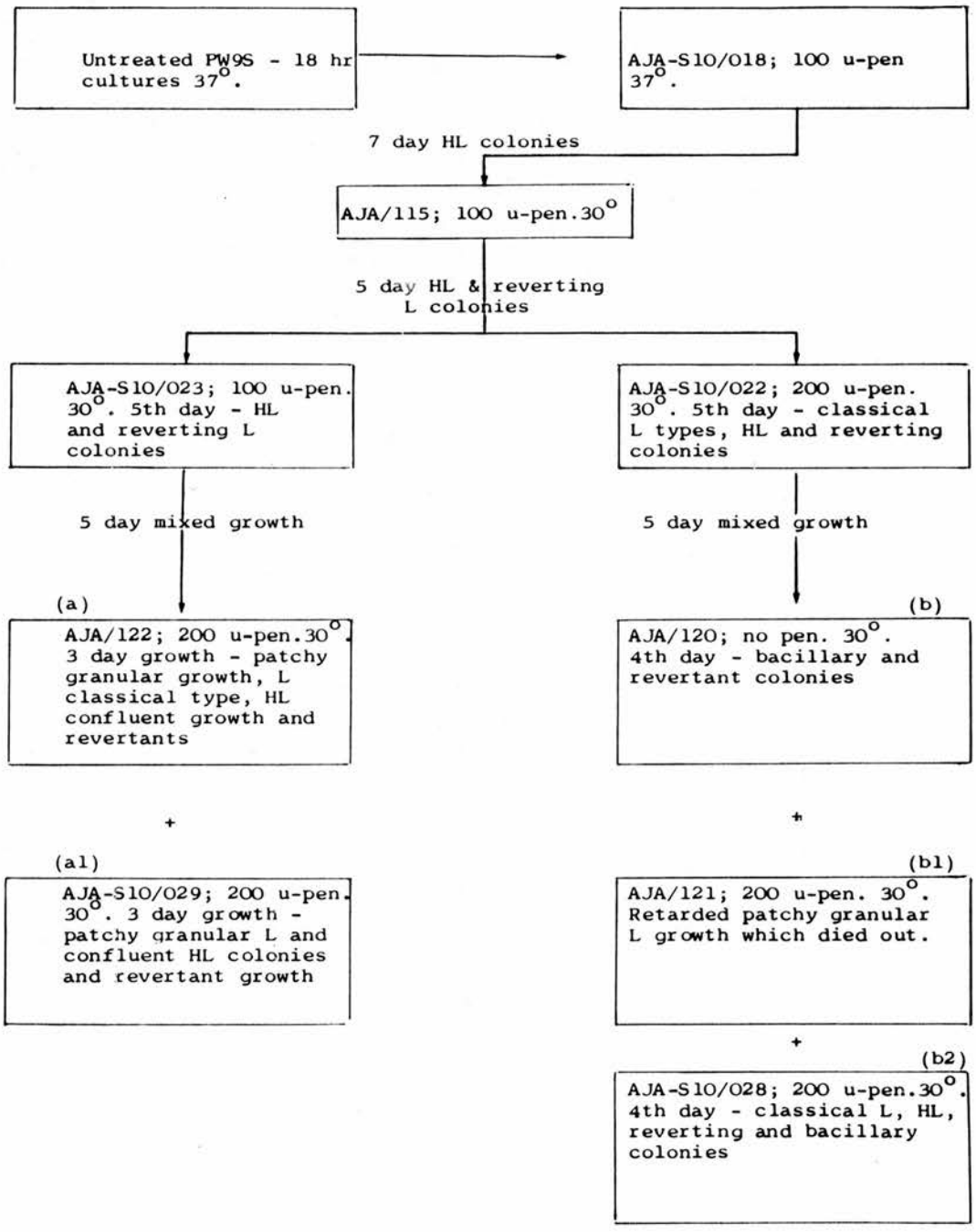
Serial subculture of L, HL and revertant colonies of 9S



See TABLE 2

TABLE 4

Serial subculture of L, HL and revertant colonies of 9S



See TABLE 2

TABLE 5

Serial subculture of L, HL and revertant colonies of 9S

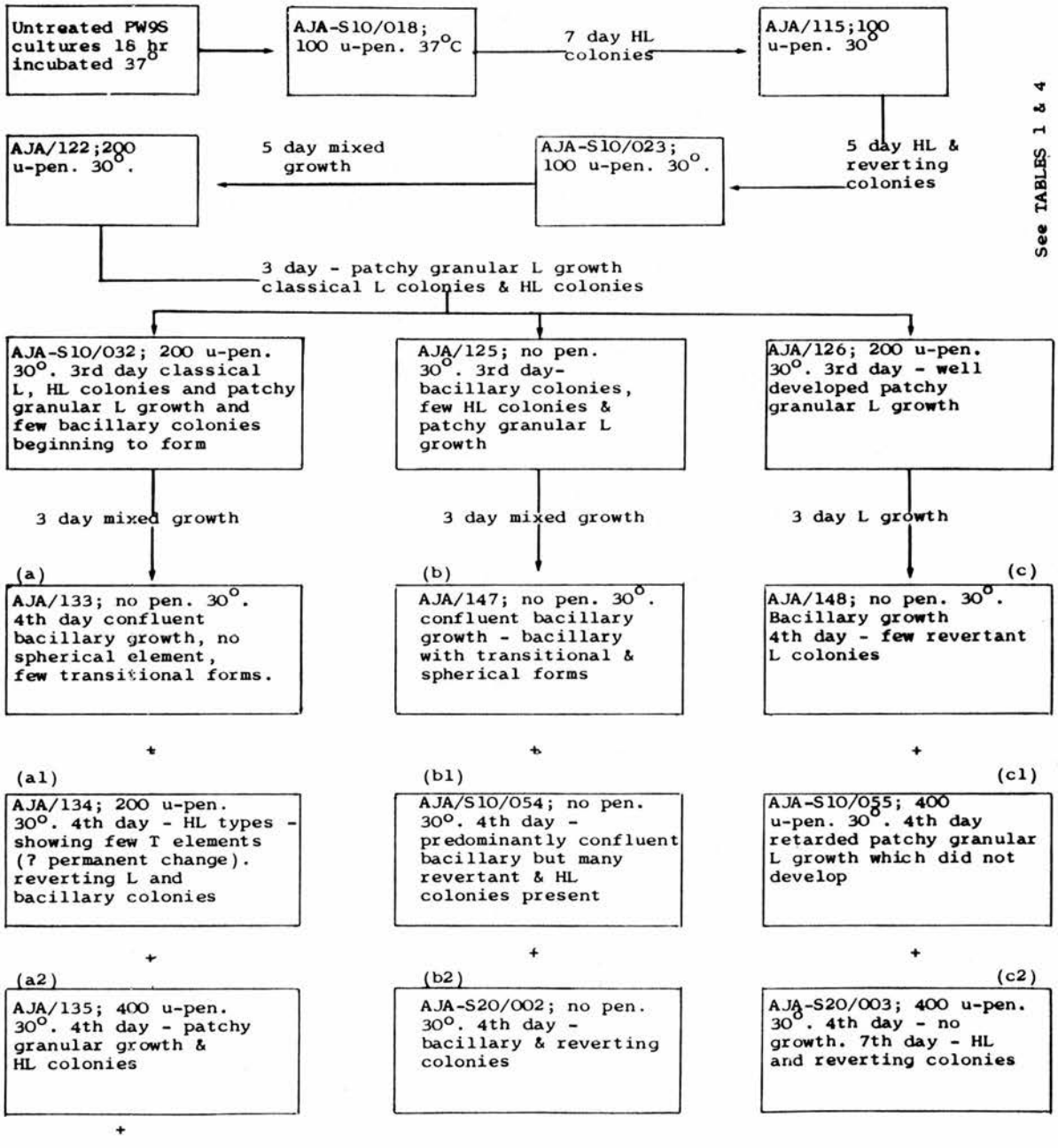


Table 5 continued

+

(a3)

AJA-S10/039; no pen. 30°. 4th day - predominantly bacillary colonies and a few revertant types.

+

(a4)

AJA-S10/040; 200 u-pen. 30°C. 4th day HL and reverting colonies

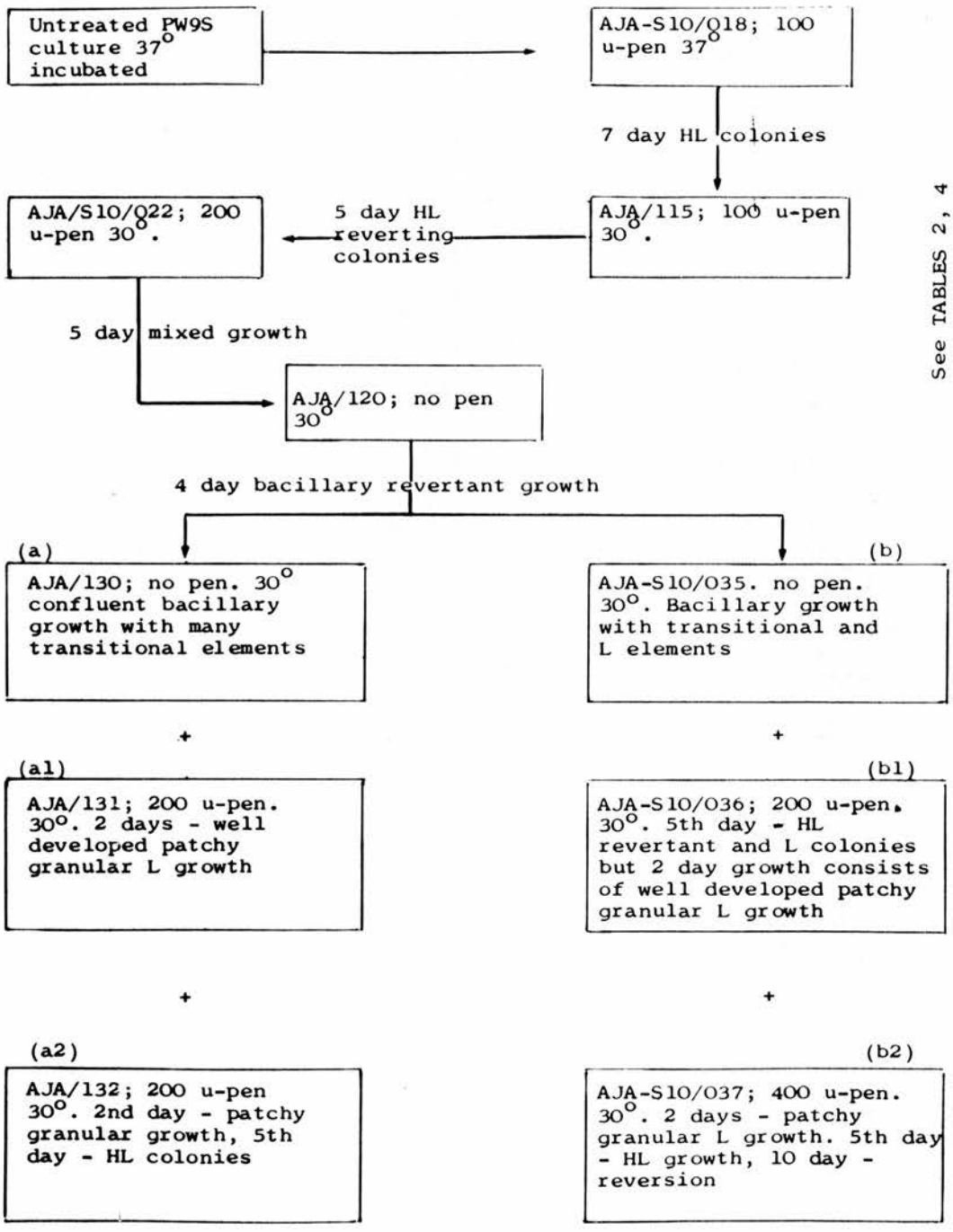
+

(a5)

AJA-S10/041; 400 u-pen, 30°. 4th day - patchy granular L growth

TABLE 6

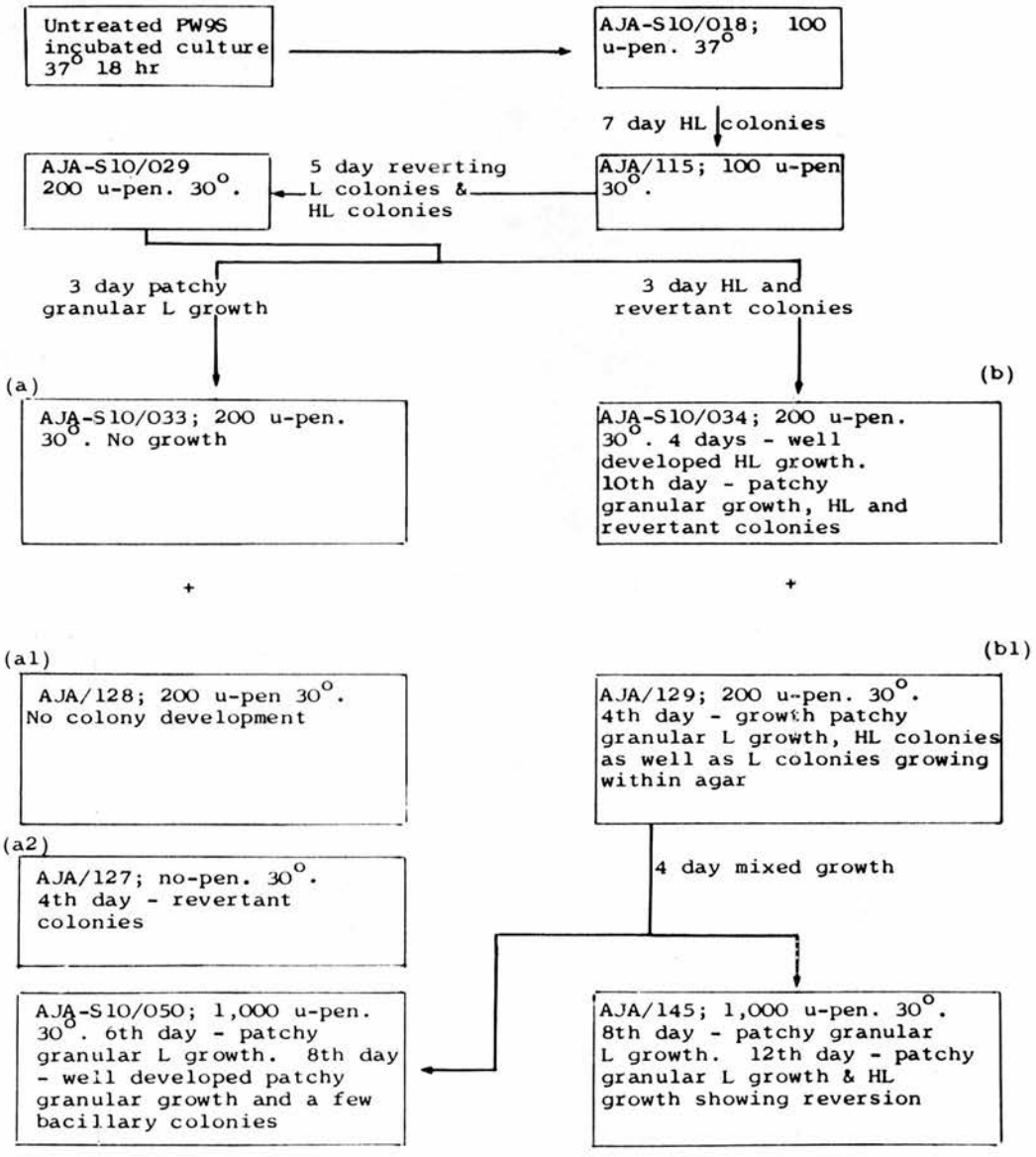
Serial subculture of L, HL and revertant colonies of 9S



See TABLES 2, 4

TABLE 7

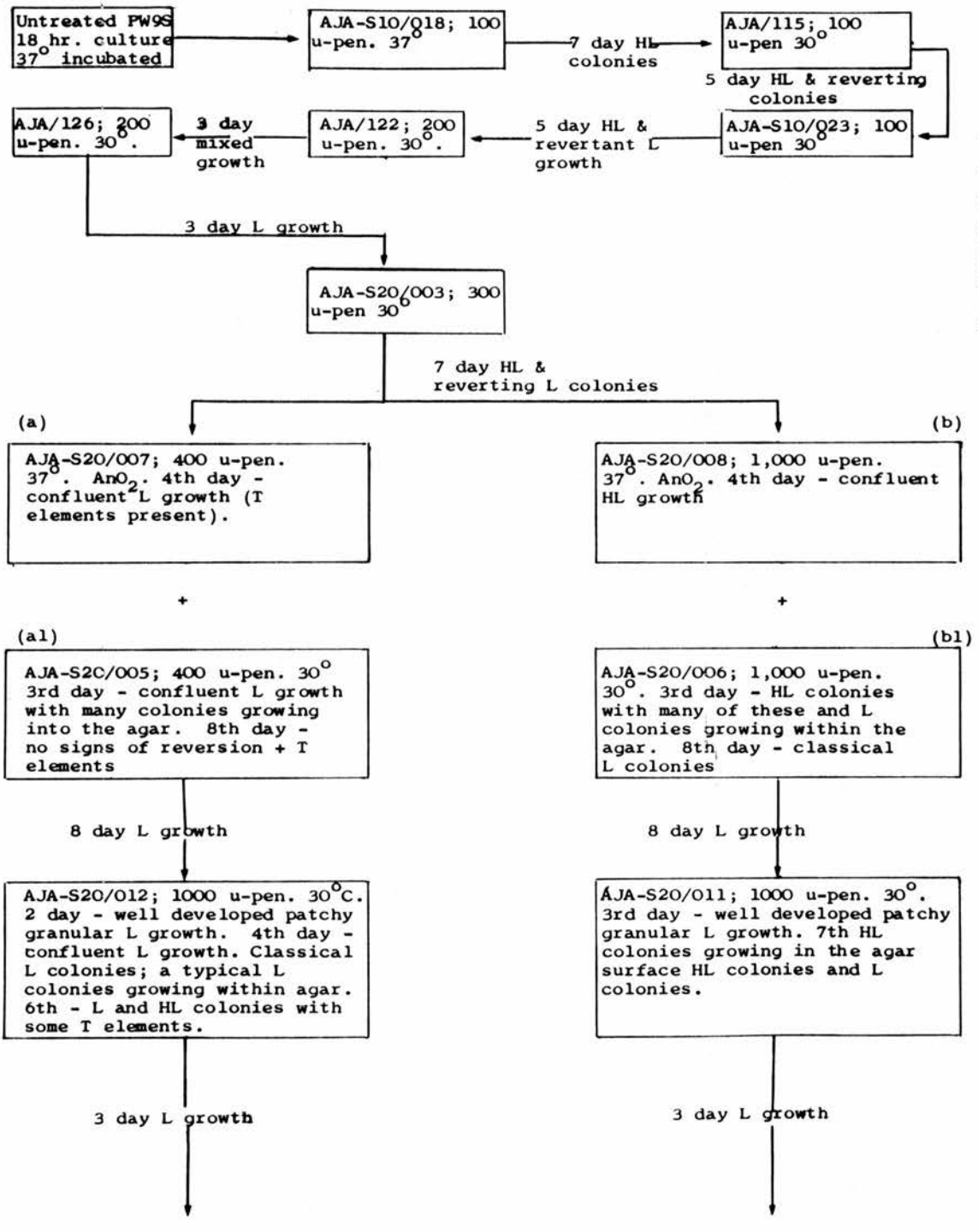
Serial subculture of L, HL and revertant colonies of 9S



See TABLES 2 & 4

TABLE 8

Serial subculture of L, HL and revertant colonies of 9S



See TABLES 2, 4, 5

Table 8 continued

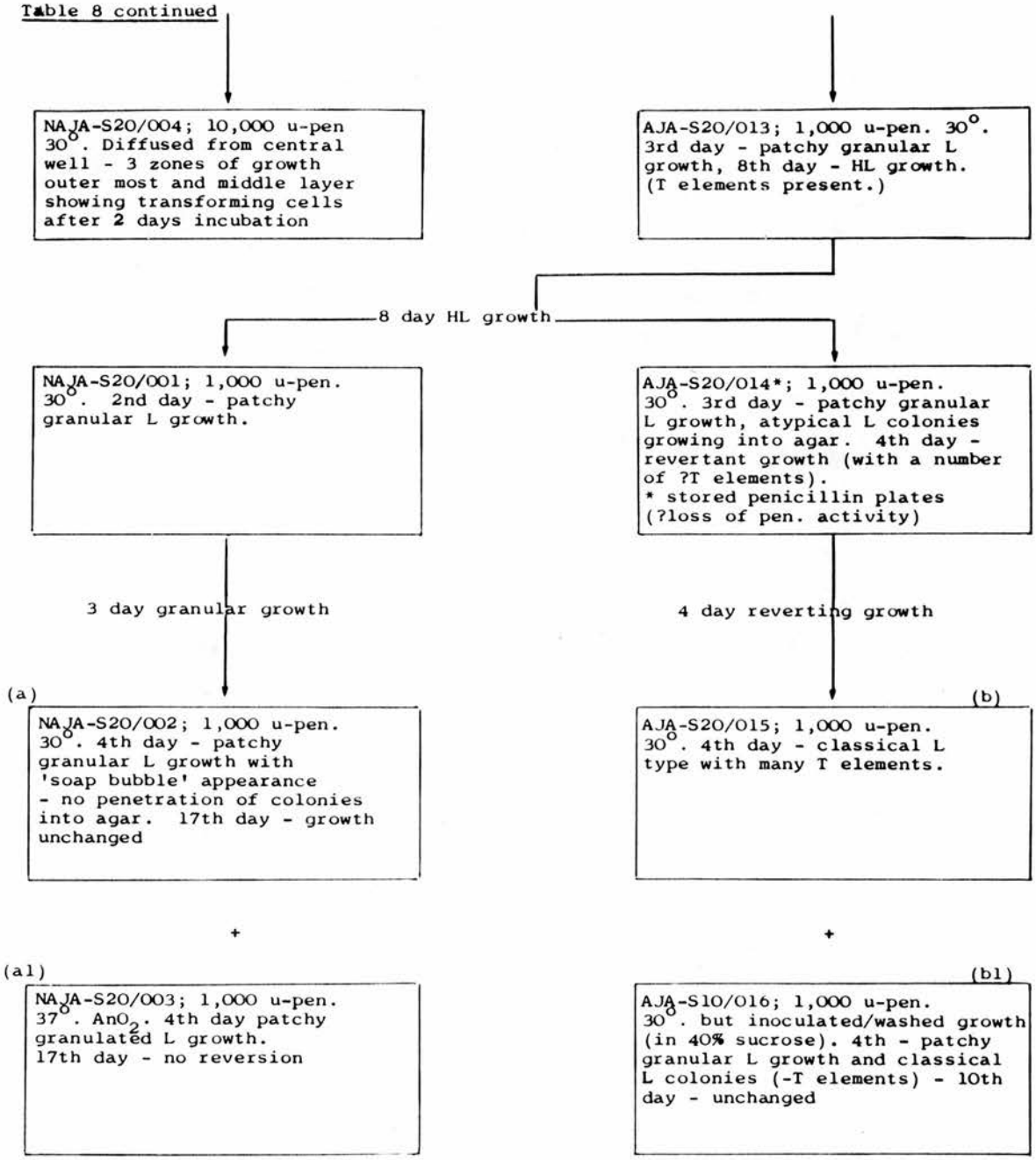


TABLE 9

Serial subculture of L, HL and revertant colonies of 9S

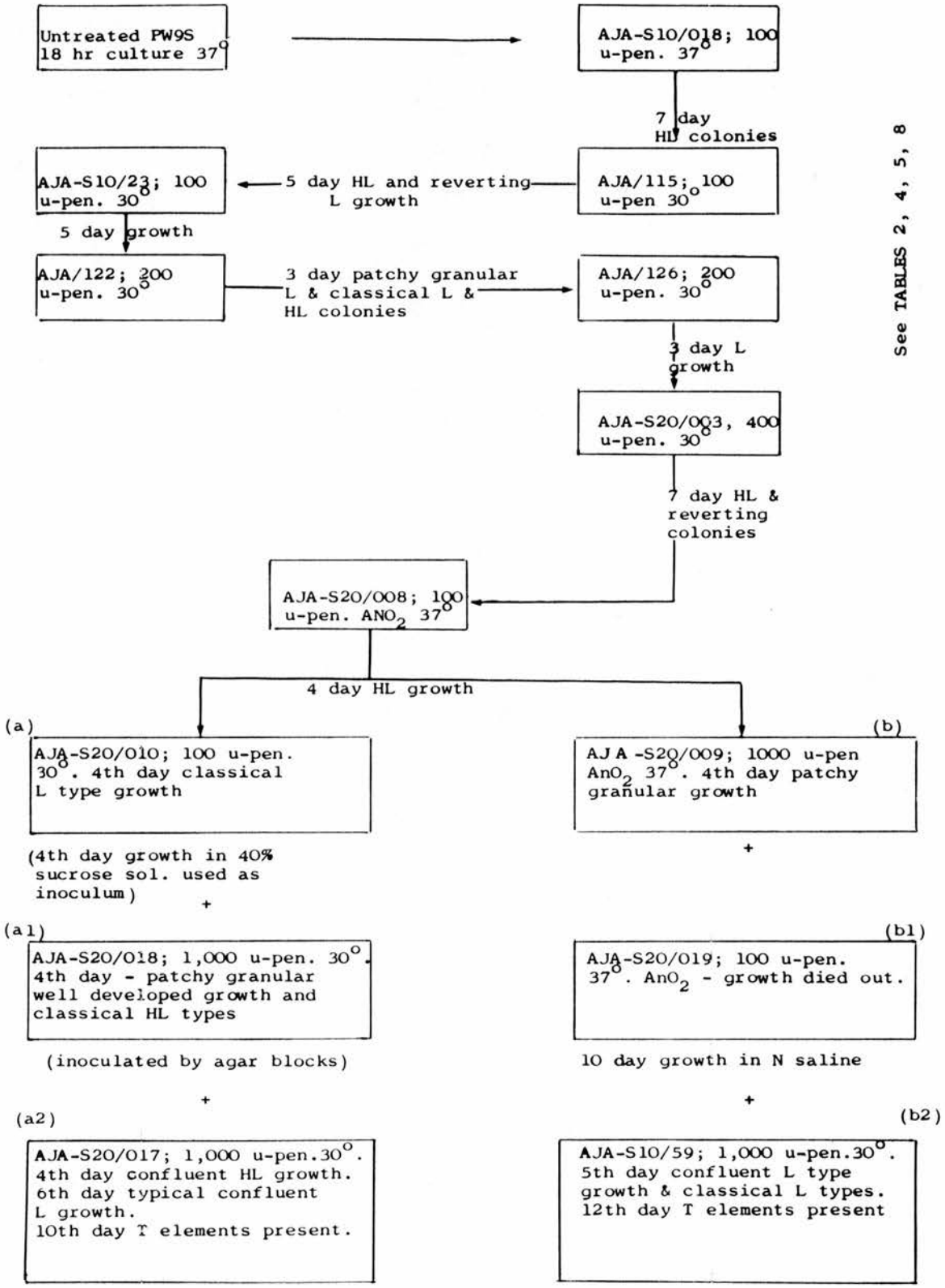


TABLE 10

Serial subculture of L, HL and revertant colonies of 9R

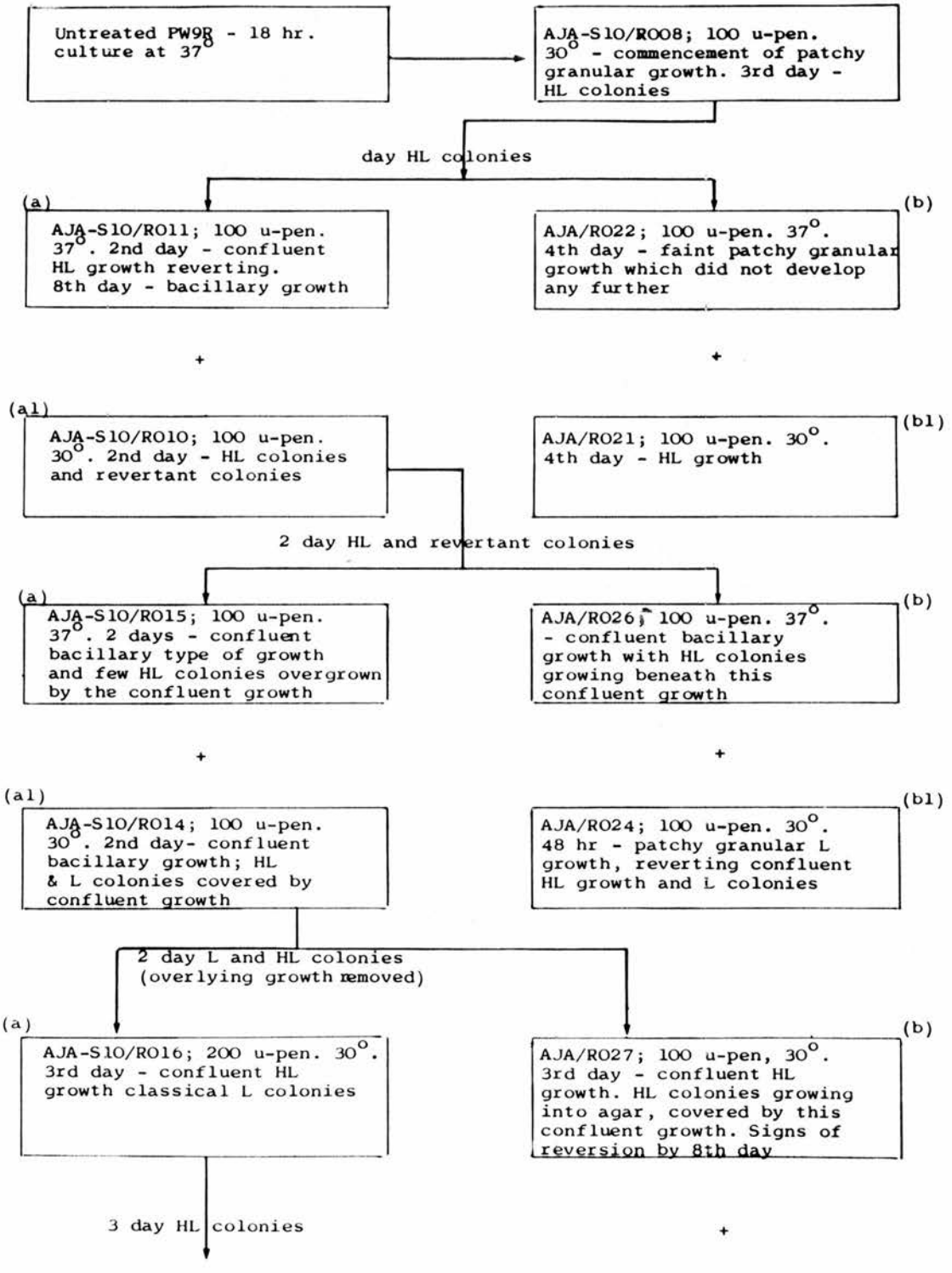


Table 10 continued

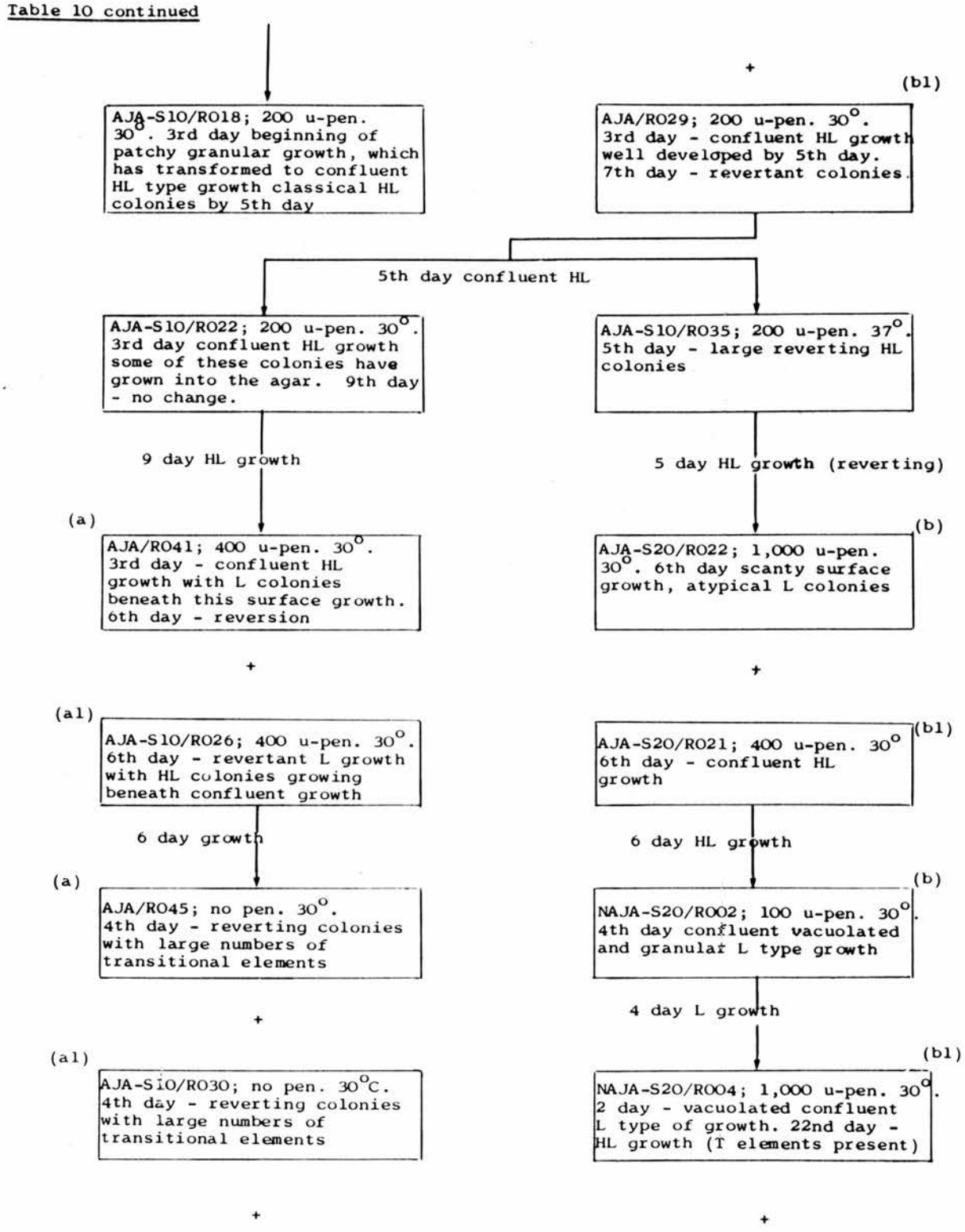


Table 10 continued

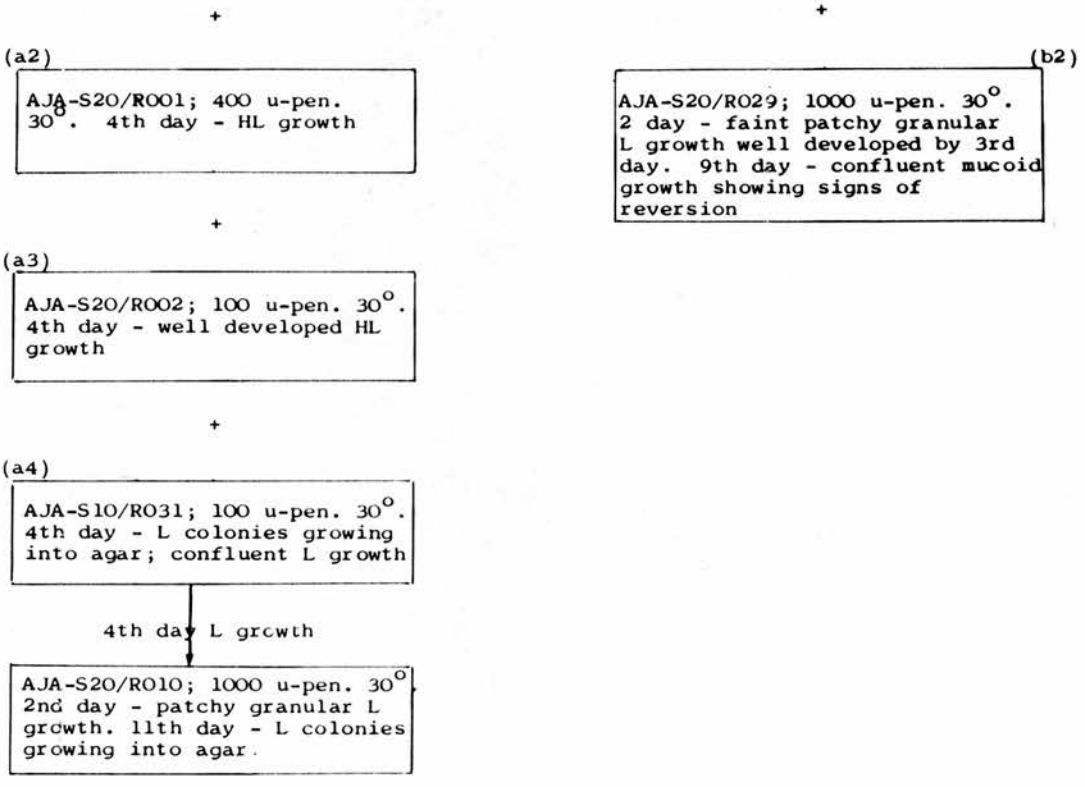


TABLE 11

Serial subculture of L, HL and revertant colonies of 9R

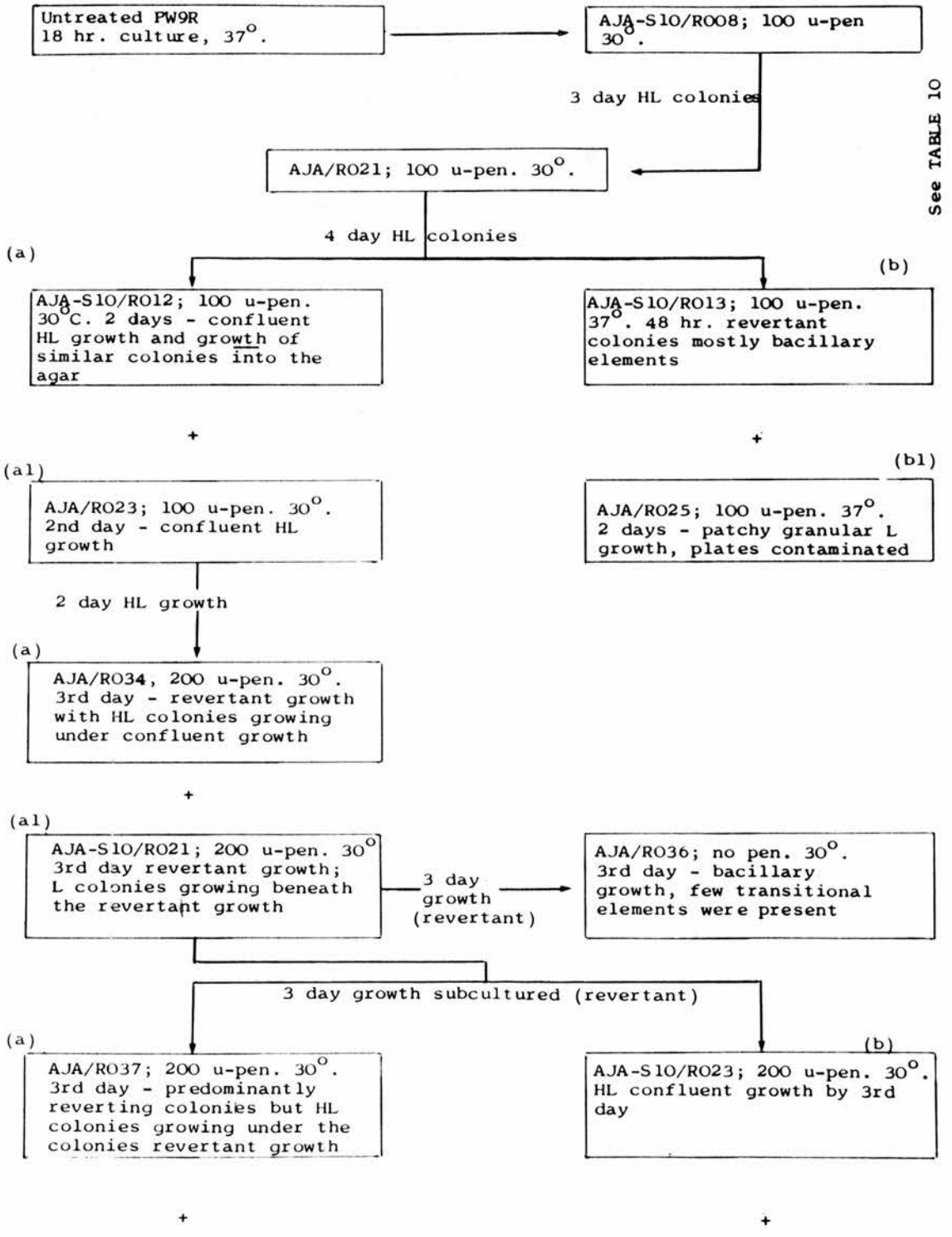


Table 11 continued

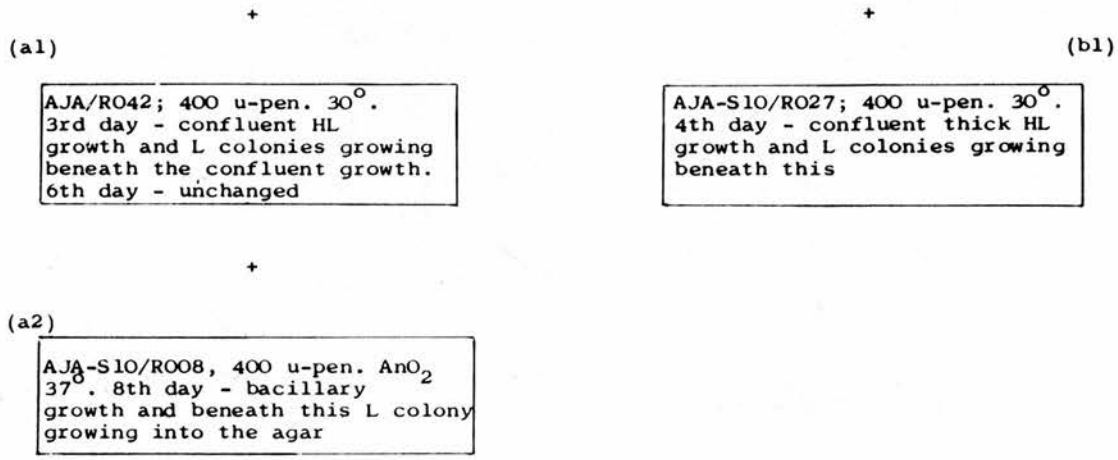


TABLE 12

Serial subculture of L, HL and revertant colonies of 9R

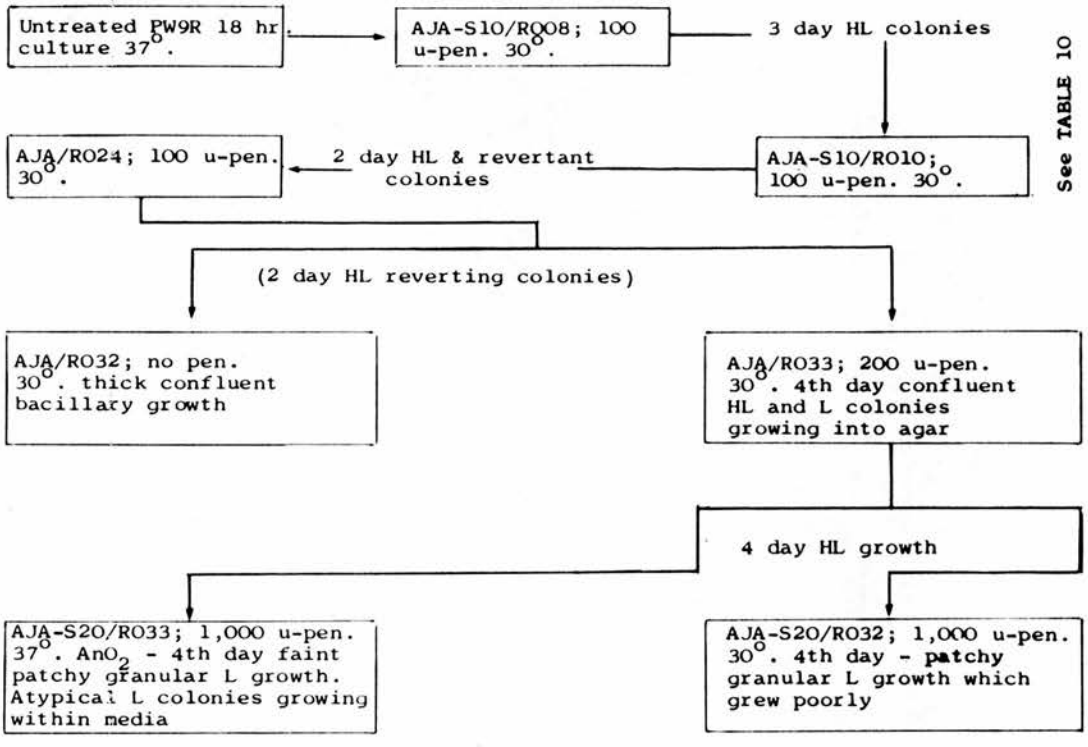
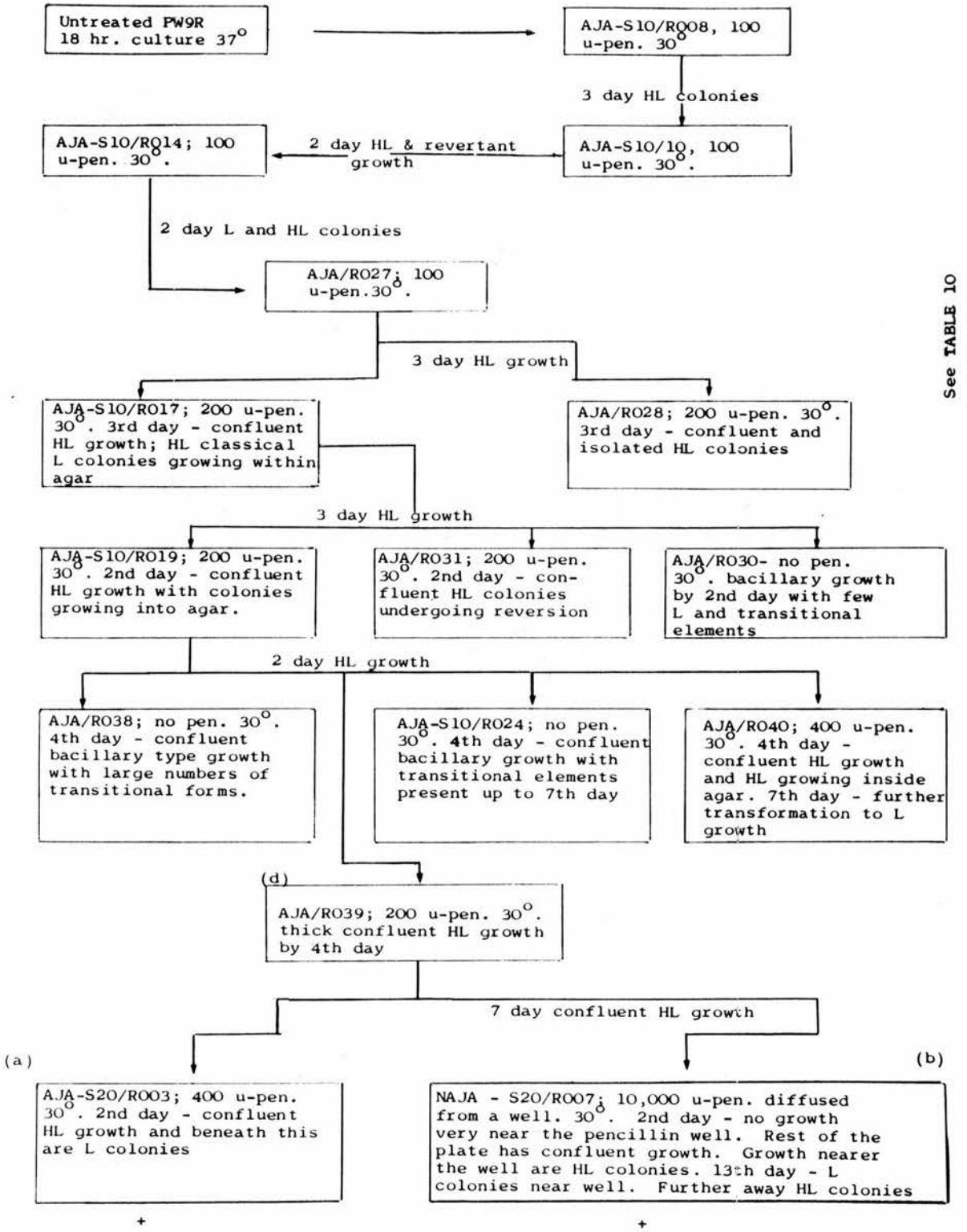


TABLE 13

Serial subculture of L, HL and revertant colonies of 9R



See TABLE 10

Table 13 continued

(a1)

AJA-S20/R035; 1000 u-pen. 37°. AnO_2. 4th day - film of bacillary growth (NB. These plates were inoculated with growth which had been stored for 2-3 weeks at 4°C.

(a2)

AJA-S20/R034; 1000 u-pen. 30°. 4th day - HL type growth. These plates were inoculated with growth that has been stored for 2-3 wks at 4°C

(b2)

NAJA-S20/R010; no pen. 30°. bacillary growth consisting mainly of coccobacillary and a few transitional elements

(b3)

NAJA-S20/R009; 15,000 u-pen. diffused from well. 30° 24 hr. - no growth immediately around well but surrounding this is a confluent revertant growth. 3rd day - the L growth near well. HL growth surrounding zone of confluent L growth.

(b4)

AJA-S10/043; no pen. 30°C. 11th day - bacillary growth with large numbers of transitional forms.

(b5)

AJA-S10/044; 1000 u-pen. gradient media. 30°. 11th day - revertant colonies with many L elements

TABLE 14

Serial subculture of L, HL and revertant colonies of 9R

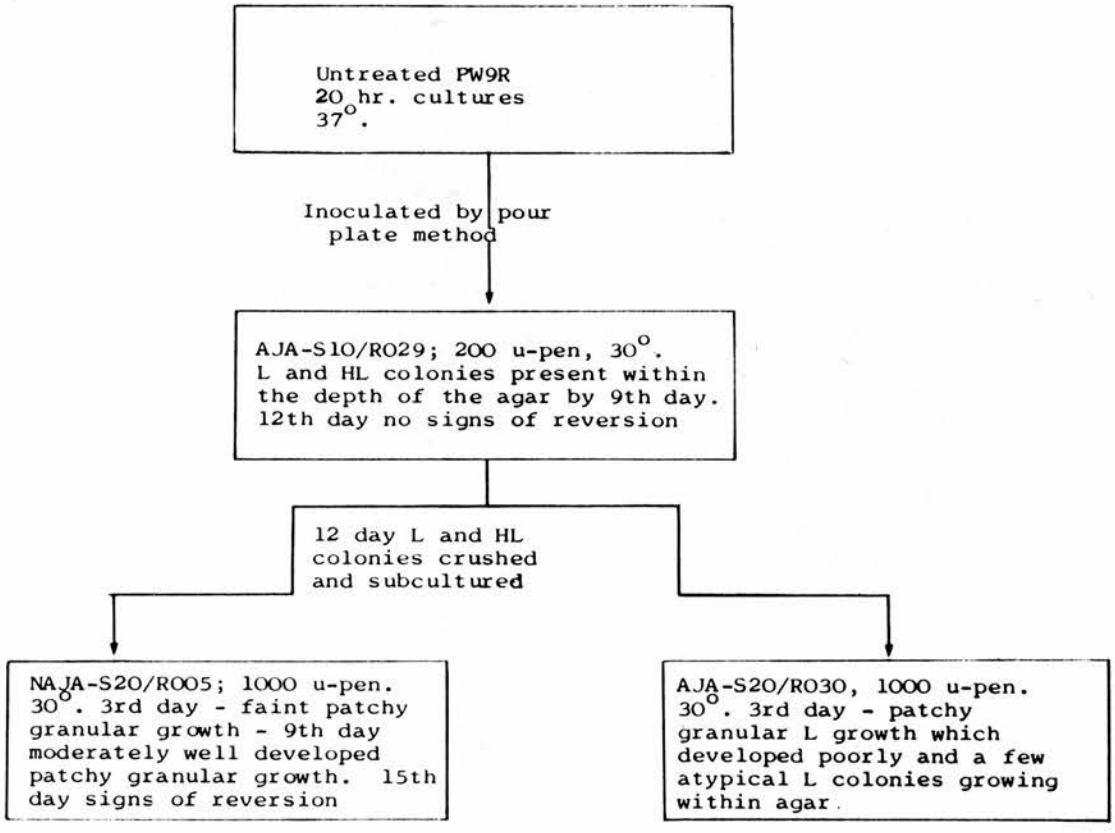


TABLE 15

Serial subculture of L, HL and revertant colonies of 9R

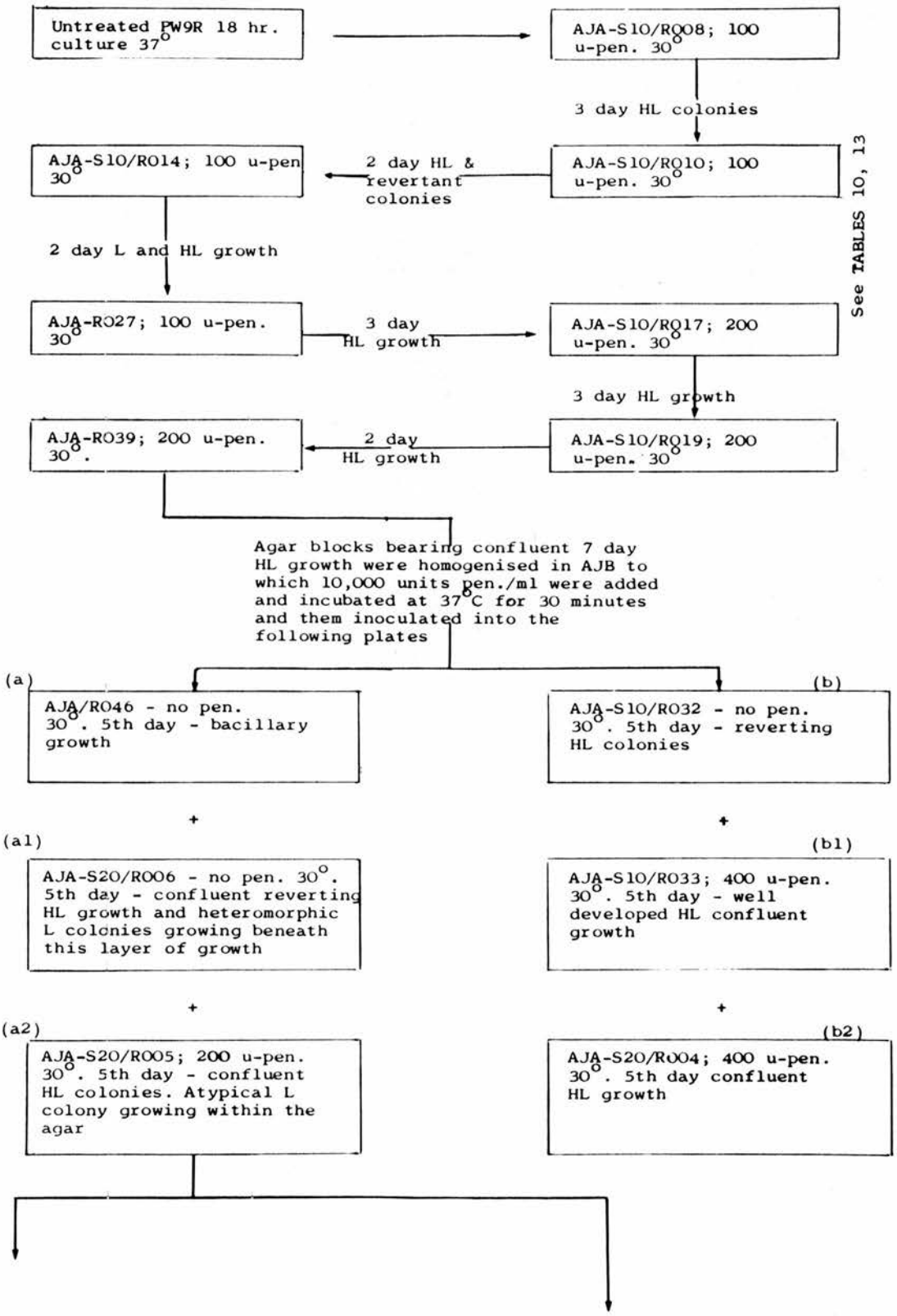


Table 15 continued

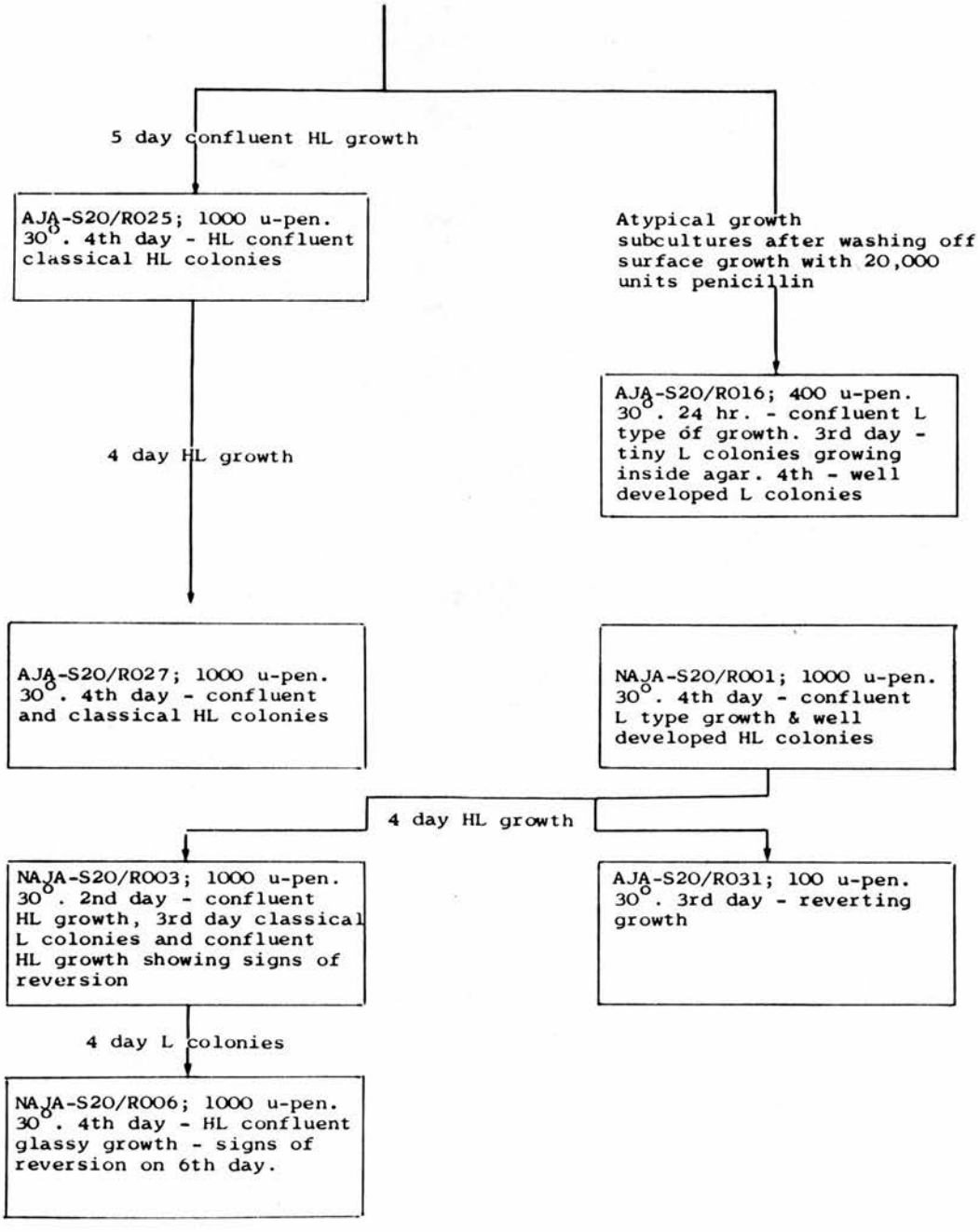
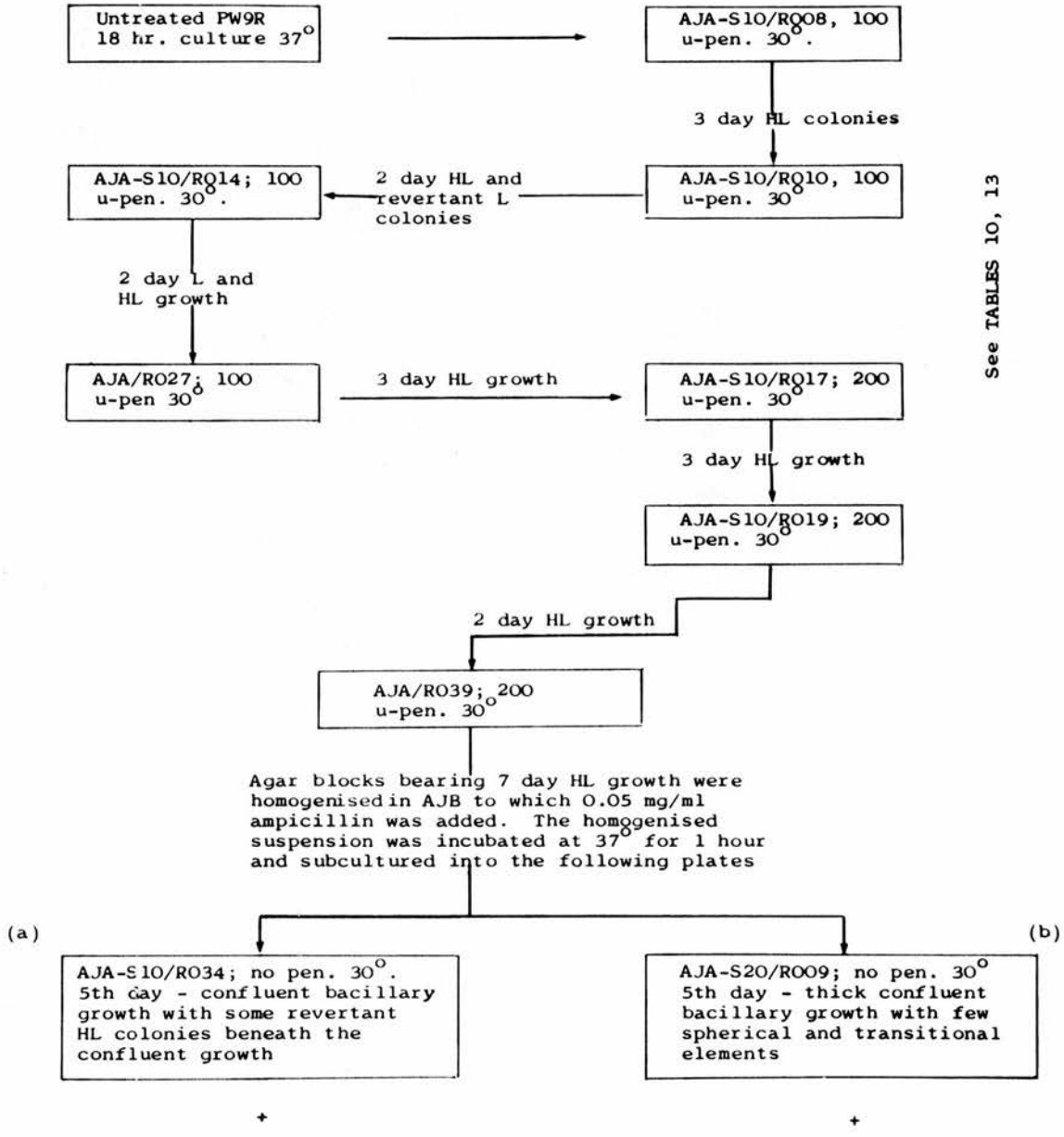


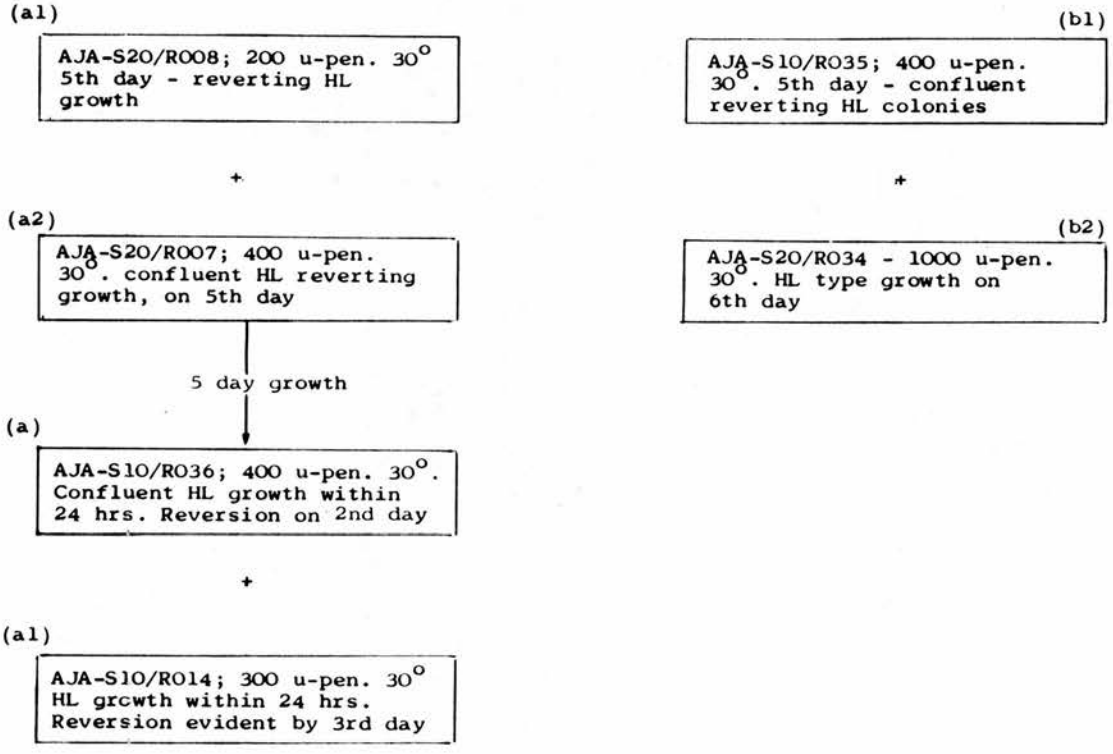
TABLE 16

Serial subculture of L, HL and revertant colonies of 9R



See TABLES 10, 13

Table 16 continued



Summary of findings from serial passage of L and heteromorphic L colonies (derived from strains 9S and 9R) on AJA medium.

1. L and heteromorphic L colonies on first subculture to AJA medium containing low concentrations of penicillin (100 units/ml) produced a patchy granular L type of growth which generally reached its optimum development by the 3rd day of incubation at 30°C. When these plates were further incubated, heteromorphic L type and revertant type of growth appeared. L and heteromorphic L colonies obtained on media with lower concentrations of penicillin, when transferred to media containing higher concentrations, also produced patchy granular L type growth, generally between the 3rd and 5th day of incubation at 30°C.

2. Only well developed patchy granular L type growth could be propagated successfully and was often difficult to obtain on AJA medium containing more than 200 units penicillin/ml.

3. Patchy granular L type growth subcultured on media containing no penicillin or serum produced revertant and bacillary colonies.

4. L colonies and heteromorphic L colonies when serially subcultured in the presence of constant amounts of

penicillin showed an increasing tendency to revert with each subculture particularly on media containing 200 units/ml or less but in serial subcultures on AJA medium containing 400 units penicillin, L and heteromorphic L colonies were still produced in addition to a small amount of revertant type growth.

5. L colonies and heteromorphic L colonies passaged alternately on high and low penicillin-AJA medium (viz. passage from 1,000 units/ml penicillin medium to 200 units/ml penicillin medium and back to 1000units/ml penicillin medium showed a marked tendency to produce the revertant type of growth. Such a procedure also favoured the development of 'persistors' (bacillary elements which show very little or no transformation even in the presence of more than 1,000 units penicillin/ml of solid medium).

6. Subculture of heteromorphic L colonies produced on media containing high concentrations of penicillin on to media with lower concentrations of penicillin promoted reversion.

7. Revertant growth and heteromorphic L colonies produced on medium containing lower concentrations of penicillin (100 units/ml) when serially subcultured on medium containing higher concentrations of penicillin (200, 400 and 1,000 units/ml) were transformed almost entirely from the transitional and bacillary forms into L elements.

This effect was more noticeable in media containing 400 or more units of penicillin/ml of the induction medium.

8. L colonies and heteromorphic L colonies produced on plates containing 400 or more units of penicillin/ml reverted less rapidly than those produced at 200 or less units of penicillin. L colonies and heteromorphic L colonies produced on AJA medium with 400 or more units penicillin/ml, when made to revert on AJA medium containing no penicillin, produced reverting bacillary growth containing more transitional and L elements than in a similar growth obtained by the reversion of L and heteromorphic L colonies produced on AJA medium containing 200 or less units of penicillin/ml.

9. When L and heteromorphic L colonies that had undergone three or more serial passages on AJA medium containing penicillin were transferred to medium containing no penicillin a few L and heteromorphic L colonies were produced in addition to the revertant bacillary growth. The revertant bacillary growth consisted predominantly of rods many of which were swollen with varying numbers of transitional forms. The L elements were usually few in number. Further subcultures on AJA medium without penicillin of the mixed growth obtained on the same medium produced an increased reversion of the transitional and L elements to the

bacillary phase. L elements were generally absent in the later type of growth thus produced.

10. In most instances, when L colonies and heteromorphic L colonies which had been produced on AJA medium containing low concentrations of penicillin and 10 per cent. of serum were transformed directly on to a similar medium but containing high concentrations of penicillin, they failed to grow. Thus, L colonies produced on 10 per cent. serum AJA medium containing 200 units of penicillin often failed to propagate when subcultured on to 10 per cent. serum AJA medium containing 1,000 units penicillin/ml, whereas L colonies and heteromorphic L colonies produced on AJA medium with 200 units penicillin/ml when passaged at least once on a AJA medium with 400 units/ml penicillin produced a good yield of L growth on AJA medium containing 1,000 units/ml penicillin. In a few instances L and heteromorphic L colonies which had been passaged serially for 3 or more times of low concentration penicillin medium (200 units/ml) could be directly transferred to AJA medium containing 1,000 units penicillin/ml. In such cases a patchy granular L type growth developed between the 6th and 8th days of incubation at 30°C.

11. The inclusion of serum in the AJA medium promoted L type growth. An increased transformation of bacillary

elements to the L phases occurred in induction medium containing serum. However, serum was also found to favour the growth of bacillary elements. Media containing 20 per cent. inactivated horse serum were superior in the propagation of L and heteromorphic L colonies to those containing 10 per cent inactivated horse serum.

12. Media containing 20 per cent. inactivated serum favoured the development of atypical L colonies which grew within the depth of the agar. These colonies were formed generally only on media that contained 400 to 1,000 units of penicillin/ml. Media containing 20 per cent. inactivated horse serum supported growth of patchy granular L type colonies better than media containing 10 per cent. inactivated horse serum or no serum at all.

13. An incubation temperature of 37°C was unfavourable for the propagation of L and heteromorphic L colonies. Reversion was more apparent and took place earlier in plates incubated at 37°C than in those incubated at 30°C. The bacillary forms inoculated on appropriate AJA medium and incubated anaerobically at 37°C were just as likely to undergo L transformation as similar cultures incubated aerobically at 30°C.

14. Reversion of L and heteromorphic colonies on AJA medium containing low concentrations of penicillin (200

or less units /ml) was more noticeable with those derived from strain 9R than strain 9S.

15. When L and heteromorphic L colonies which had been made to revert to the bacillary forms were subsequently subcultured to induction media containing penicillin, they were found to resist L transformation. Even after several passages in penicillin media a large number of 'persistors' were present and these often overgrew the other colony types (L and heteromorphic L growth).

16. Continued passage of L and heteromorphic L colonies on media containing 1,000 or more units penicillin/ml medium produced a growth which consisted of abnormal forms - large fusiform and bulbous elements, aster-like bodies and swollen branching filamentous forms. These elements were difficult to differentiate from the transitional elements except that (a) they were only produced on 20 per cent. serum-AJA and -NAJA media containing very high concentrations of penicillin (1,000 or more units/ml) and (b) similar elements were found in L growth adapted to grow in liquid media.

These forms were occasionally produced on AJA medium containing 10 per cent. serum and 1,000 units penicillin/ml and sometimes on 20 per cent. serum AJA medium containing 400 units penicillin/ml. These forms were provisionally designated as 'T' elements

(see Plate 30)

17. Another type of element found in L and heteromorphic L growth derived from strain 9R was a giant coccobacillary bipolar form found in association with large round pale-staining bodies and large spherical elements (see plates 31,32 & 33). It can be seen from these micrographs that these coccobacillary forms tend to occur in small clusters and also show chain formation. The large more intensely stained spherical bodies tend to occur in small clusters and generally show a condensation of cytoplasmic contents towards one end of the limiting membrane. A few distorted large round pale-staining bodies are also faintly visible. There were also a few small and medium sized spherical bodies. These elements were found in both AJA and NAJA plates containing 20 per cent. inactivated horse serum and penicillin concentrations of from 200-1,000 units/ml. The appearance of these giant coccobacillary forms has occurred in subcultures after heteromorphic L growth has been exposed to very high concentrations of penicillin or ampicillin.

18. Heteromorphic L growth that had been exposed to very high concentrations of penicillin (20,000 units/ml) in AJB and then subcultured on to media containing 200 and 400 units penicillin/ml produced heteromorphic L growth only, and further subcultures of this heteromorphic L growth on AJA medium containing 1,000

units penicillin/ml failed to produce L growth, indicating that a number of resistant forms had emerged that did not readily transform. Similarly, when heteromorphic L growth that had been exposed to the action of ampicillin (0.05 mg/ml) in AJB was subcultured on to 20 per cent. serum AJA medium containing 200 units penicillin/ml it produced on the 5th day of incubation a heteromorphic L growth which was of revertant type (i.e. containing large numbers of bacillary elements in addition to the transitional and L elements); similar findings were observed on 10 per cent. serum AJA medium containing 400 units penicillin/ml; but in 20 per cent. AJA medium containing 400 and 1,000 units penicillin/ml a well developed heteromorphic L growth was present on the 5th and 6th day of incubation, but no L colonies were produced.

19. NAJA medium was superior to AJA medium for the passage of L and heteromorphic L colonies. Reversion was slower on NAJA medium than on AJA medium.

20. L colonies passaged on AJA medium containing 1,000 units penicillin/ml and transferred to NAJA medium containing the same concentration of penicillin produced a well developed confluent growth which, under the x5 objective of the light microscope showed a vacuolated type of growth.

21. Heteromorphic L colonies subcultured on 20 per cent.

serum NAJA diffusion medium (10,000 and 15,000 units penicillin diffused from a central well in the agar) produced two zones of growth. There was no growth of any elements in a zone 10 mm. wide from the penicillin wells. Immediately surrounding this zone of no growth was a ring of confluent L growth (about 5-10 mm. wide) and this merged with the outer ring of heteromorphic L growth. The width of the L growth and heteromorphic L growth was determined by the amount of penicillin in the well and the period taken by the penicillin to diffuse into the surrounding medium. Thus, on NAJA medium in which 10,000 units of penicillin was placed, a plate incubated for 2 days showed growth which was still in the process of L transformation whereas on the 7th day the L and heteromorphic L growth had achieved their maximum development. On the other hand, with 15,000 units penicillin in the central well, L growth was already evident by the 3rd day of incubation.

22. As a result of continued passage, L colonies and heteromorphic L colonies could be transferred from one medium to another by inoculating the washed growth. L and heteromorphic L colonies washed with 40 per cent. sucrose or normal saline were equally effective in propagating growth suggesting that (a) large numbers of the L elements occur on surface of the plate and (b) many of the L elements have acquired osmotic resistance

indicated by the fact they could be transferred to new media via normal saline washings.

23. There was evidence to indicate that accidental plate contaminants (yeast colonies in particular) promoted reversion to a greater degree than occurred in uncontaminated plates containing the same induction medium. These findings are casual observations made from a comparison of duplicate plates.

INDUCTION OF L FORMS IN LABORATORY STRAINS (9S AND 9R) WITH GLYCINE

Overnight concentrated PW cultures of 9R were plated out on the following media - AJA medium (with and without serum) not containing glycine, AJA medium containing 1.5 and 3.0 per cent. glycine but with no added serum and on 10 per cent. serum AJA medium containing 1.5 and 3.0 per cent. glycine. All the inoculated plates were incubated at 30°C. On AJA medium (not containing serum) in which 1.5 per cent. glycine had been incorporated, a dry confluent bacillary type of growth developed within 48 hours of incubation. This growth consisted primarily of swollen medium sized bacillary rods, moderate numbers of longer and shorter rods and filaments and a few 'yeast-like' forms. Also present were a few L elements (mainly large and medium spherical bodies and a few large round pale-staining forms). When the plates were re-examined on the

7th day of incubation the growth consisted essentially of the same elements found in the 48 hour growth but, in addition, a few tiny spherical elements were present. In another experiment using the same medium, containing 1.5 per cent. glycine, the period of observation was extended to 9 days. The 9 day growth showed numerically more L elements than the 7 day growth. In AJA media (not containing serum) with 3.0 per cent. glycine the growth was extremely poor. These plates, even after 9 days' incubation, had only developed a faint patchy granular L type growth in a small area of the plate, but 3-4 small irregularly shaped colonies were present measuring less than 0.5 mm in size and having a velvety wrinkled appearance (see plate 34). On 10 per cent. serum AJA medium the inducing effects of glycine were more marked. On 10 per cent. serum AJA medium containing 1.5 per cent. glycine a very slimy and firmly adherent growth was present when the plates were examined on the 6th day of incubation. This growth consisted of transitional filamentous forms (mostly medium, small and tiny spherical elements with a few large spherical elements and large round pale-staining bodies), and irregularly shaped bodies. There were large numbers of extracellular granules staining blue with Dienes' stain. Occasionally swollen rod forms were encountered. This growth represented a well developed confluent heteromorphic L type growth. By the 9th day of

incubation there were signs of reversion. The growth at this stage of incubation consisted predominantly of very long swollen rods, yeast-like bodies, short swollen filamentous forms (all these three types are considered to be early transitional forms) and small numbers of tiny and small spherical elements. There was a notable absence of large round pale-staining bodies and long filamentous forms.

On 10 per cent. AJA medium containing 3 per cent. glycine, a well developed patchy granular L growth was present when the plates were examined on the 9th day. In addition 5-10 irregularly shaped colonies were present similar to those produced on AJA medium containing 3 per cent. glycine but not serum. These irregularly shaped colonies, when stained by Gram's method, showed a mass of nondescript pinkish material with a few Gram negative coccal forms, but by Dienes' staining method the colonies were found to be composed of L elements; no transitional or bacillary forms were present. The L elements consisted predominantly of large round pale-staining bodies, large and medium sized spherical elements and some tiny spherical elements.

The control AJA medium did not produce any L transformation. The control AJA medium containing serum showed a larger number of filamentous forms than that not containing serum.

With strain 9S, glycine produced similar

transformative changes. The colony morphology exhibited by altered forms of the two laboratory strains 9S and 9R in media containing glycine were however strikingly dissimilar.

Overnight concentrated PW cultures of 9S were plated out on 10 per cent. serum AJA medium containing 1.5 and 3.0 per cent. glycine, and on a control AJA medium containing only 10 per cent. horse serum. All inoculated plates were incubated at 30°C. A thin film of confluent growth developed on the 10 per cent. serum AJA medium containing 1.5 per cent. glycine within 24 hours. When these plates were re-examined on the 4th day the previously translucent growth had become opaque and was of a moist consistency. Microscopically this growth showed mostly large numbers of L elements (spherical elements of various dimensions, large round pale-staining bodies, irregularly shaped forms and intracellular and extracellular dark blue staining granules (Dienes' stain). These L elements constituted almost half the cells found in the stained cultures. The remaining 50 per cent. consisted predominantly of medium sized bipolar rods and coccobacillary forms, and a few transitional filamentous forms. These microscopical findings suggested that the growth formed on the above plates was possibly a heteromorphic L growth beginning to revert. When the plates were examined on the 17th day of incubation the confluent moist growth was in the

process of reversion and the growth at this stage of incubation consisted primarily of short rods, coccobacilli and a small number of spherical elements. Over the 17 days of incubation the growth never became slimy or adherent to the medium as with strain 9R. The bacillary forms of 9S plated out on 10 per cent. serum AJA medium containing 3 per cent, glycine produced a faint patchy granular L type growth by the 4th day which consisted mainly of large round pale-staining bodies, many of which appeared to have been grossly distorted giving a filamentous appearance (see plate 35). There were a few spherical elements of varying sizes. The large round pale-staining bodies were very coarsely granular. No transitional, tiny spherical or bacillary elements were seen in any of these cultures. By the 14th day of incubation the growth had become more opaque and profuse and microscopically consisted of almost the same elements found on the 4th day, but there were both quantitative and qualitative differences, there being proportionately more spherical elements and minimal distortion of the large round pale-staining bodies. Furthermore, in these large round pale-staining bodies there were extremely large dense granules and these were also present in some of the large and medium sized more lightly stained spherical elements (see plate 36). There was an almost complete absence

of any bacillary or transitional elements in the 14th day growth. The control AJA medium containing 10 per cent. horse serum produced a thick, dry, confluent bacillary growth by the 4th day, and this consisted of long rods, turgid medium sized rods and, to a lesser extent, short rods and coccobacilli. The growth examined on the 14th day consisted essentially of the same elements found in the 4th day growth except that a few transitional filamentous forms were also present.

PROPAGATION OF GLYCINE-INDUCED L FORMS (STRAIN 9R)

A 9-day glycine-induced heteromorphic L growth (produced on AJA medium containing 1.5 per cent. glycine) was subcultured on 10 per cent. serum AJA medium without any inducing agents and on the same medium to which had been added either (a) 1.5 per cent. glycine or (b) 3 per cent. glycine and incubated at 30°C. On AJA medium containing no inducing agent a very thick opaque confluent growth was present after 3 days' incubation. Gram's and Dienes' stained preparations of the culture showed short stout rods, and turgid coccobacillary forms. Small to moderate numbers of transitional filamentous forms, medium and large spherical bodies, and coccal bodies were also present. When the plates were examined on the 9th day of incubation the reversion changes were more pronounced in that the growth did not show any L

elements but, on the other hand, there was an increase of the filamentous forms.

On 10 per cent. AJA medium containing 1.5 per cent. glycine, a very slimy adherent growth developed by the 3rd day. The microscopic elements comprising this growth were mainly short and medium sized swollen rods, turgid coccobacilli and a few short filaments. Also present in small but perceptible numbers were tiny and small spherical elements and occasional large spherical elements and large round pale-staining bodies. The colonial morphology remained unaltered when the plates were examined on the 9th day of incubation and at this time the slimy heteromorphic confluent L growth contained an admixture of pleomorphic bacilli, large numbers of tiny spherical forms and moderate numbers of small and medium sized spherical bodies. The microscopical findings after 3 and 9 days show that at 9 days the growth was still in the transformative stage and reversion to the bacillary phase was not evident. On 10 per cent. serum AJA medium containing 3 per cent. glycine, a confluent granular L type growth was present after 3 days. This was non-slimy, and microscopically was seen to consist predominantly of large round pale-staining bodies and distorted forms of these; both the distorted and undistorted forms contained large numbers of coarse granules. There were present also a few to moderate numbers of spherical elements of various sizes. The

microscopical appearance of this 3-day growth was not unlike the 4-day L type growth obtained by plating out PW cultures of 9S on 10 per cent. serum AJA medium containing 3 per cent. glycine (see page 278) In addition to the confluent granular L type growth, many tiny well defined compact L colonies grew within the depth of the agar. These colonies did not have the classical "fried egg" morphology. With further incubation (i.e. up to the 9th day of incubation) the numbers of colonies growing within the depths of the agar increased. The surface granular L growth was unaltered and microscopically no bacillary or transitional elements were observed in the 9-day growth, indicating the absence of reversion.

INDUCTION OF L FORMS BY THE COMBINED ACTION OF GLYCINE AND PENICILLIN

The purpose of these experiments was to find out whether two chemically different substances (glycine and penicillin), which interfere with cell wall synthesis, would, when used together, exhibit a synergistic effect in transforming bacillary forms to the L phase. In these experiments when both the inducing agents were used together, the concentration of penicillin incorporated into the induction medium was kept to a low level (50 units ml penicillin) so as not to completely inhibit growth. Concentrated PW cultures of 9S and

9R were plated out on the following media:

(a) AJA medium containing no serum but having 1.5 per cent. glycine

(b) AJA medium containing 10 per cent. horse serum and 1.5 per cent. glycine

(c) AJA medium containing 10 per cent. horse serum, 1.5 per cent.glycine and 50 units penicillin/ml.

(d) AJA medium containing 10 per cent. horse serum, 3.0 per cent. glycine and 50 units penicillin/ml.

(e) AJA medium containing 10 per cent. horse serum and 50 units penicillin/ml.

(f) AJA medium containing 10 per cent. horse serum only.

Inoculated plates were incubated at 30°C.

Strains 9R on AJA medium containing only 1.5 per cent. glycine had produced a confluent bacillary type of growth when examined after 2 days. This 48-hour growth consisted predominantly of long and medium sized rods with moderate numbers of short filaments and very few L elements. Five days later the growth consisted of the same elements except that there was an increase in the number of L elements and a few long serpentine forms had made their appearance.

On AJA medium containing 1.5 per cent. glycine and 10 per cent. serum the growth produced after 2 days

was extremely slimy and adherent and consisted of a large number of L elements (spherical elements of all sizes) and transitional as well as bacillary forms. The 7-day growth on this medium showed the same elements as the 2-day growth, but this time there was a considerable reduction in the number of bacillary elements. On AJA medium containing 10 per cent. serum, 1.5 per cent. glycine and 50 units/ml penicillin the 48 hour growth appeared as a confluent film of bacillary growth but when examined under a x5 objective it also had a slightly granular appearance. The microscopic composition of this 48-hour growth (plate 37) consisted mainly of transitional filamentous forms, small numbers of L elements (10-15 per cent.) and about an equal proportion of bacillary rods. By the 7th day of incubation this growth had become opaque and less moist and more profuse. This 7-day growth was an admixture mainly of L and transitional filamentous forms. The rod forms constituted less than 1.0 per cent. of the total cellular elements. A patchy granular L type growth was produced in 48 hours by strain 9R plated out on AJA medium containing 10 per cent. serum, 3.0 per cent. glycine and 50 units/ml penicillin. A few small L colonies were also growing within the depths of the agar. The growth of both the surface and deep colonies was however, very scanty. No further colonial development was noticed when the plates were

re-examined on the 7th day of incubation.

On AJA medium containing 10 per cent. serum and 50 units/ml penicillin a confluent mucoid growth and well isolated large colonies with a dense dark central core similar in appearance to a reverting L colony (see plate 29) developed after 2 days' incubation. Microscopically both the confluent growth and the isolated colonies could be considered as heteromorphic growth because both consisted of 50-60 per cent. of L elements of various sizes and less than 10 per cent. bacillary rods. The remaining elements were transitional forms (many abnormal filamentous forms). There were many distorted irregularly shaped L forms. When the growth was examined on the 7th day of incubation many of the colonies were coalescing; some appeared as revertant L type colonies and many areas of the plate had a greyish white confluent growth. Microscopically there was a four- to five-fold increase in the number of bacillary elements compared with the 48 hour growth. Transitional and L elements were in almost equal proportions. None of the L elements was demonstrable by Gram's staining technique. Colonies stained by Dienes' method showed the predominating L elements to be the small and medium sized spherical elements. Large numbers of tiny spherical elements were also present. There was a tendency for some of the larger

L elements to occur in clusters.

On AJA medium containing no inducing agent, cell transformation did not occur. The 7-day growth examined microscopically was found to consist principally of coccobacillary forms similar to those shown in plate 27.

Parallel experiments were carried out with strain 9S. On AJA medium containing only 1.5 per cent. glycine, strain 9S produced at 48 hours a moist opaque and confluent growth which consisted of small rods, coccobacillary forms and a few short and long filaments with small numbers of L elements. With further incubation up to 7 days, the confluent growth became more profuse but the microscopical composition was not significantly altered. Strain 9S was not examined on AJA medium containing 10 per cent. serum and 1.5 per cent. glycine. On AJA medium containing 10 per cent serum, 1.5 per cent. glycine and 50 units/ml penicillin, strain 9S produced a thin film of patchy granular L type growth. Of the two plates inoculated, visible growth was present on only one. This 48-hour growth showed mainly L elements (spherical elements varying in sizes from tiny forms to the large varieties and large round pale-staining bodies) and granules. There were also large numbers of transitional rod and filamentous forms. A large number of these filamentous forms were beaded as shown in plate 37. There was a complete absence of bacillary forms. By the 7th day the growth was profuse, a moist and confluent and, in addition, a

large number of discrete heteromorphic L colonies with a classical morphology was also present. The moist confluent growth was also a heteromorphic L growth showing large numbers of filamentous forms and L elements and small numbers of long rods. The large and medium sized spherical bodies appeared in small clusters and groups. Also present in appreciable numbers were very coarse extracellular granules staining blue by Dienes' stain. On AJA medium containing 10 per cent. serum, 3 per cent. glycine and 50 units/ml penicillin, strain 9S produced a very faint patchy granular L type growth in a few areas of the plate. This growth consisted entirely of L elements many of which were distorted resembling the forms seen in plate 35. By the 7th day of incubation the growth was more extensive and slightly more profuse and opaque. Microscopical examination showed the growth to consist entirely of L elements (predominantly medium, small and tiny spherical elements) with moderate numbers of large distorted bodies and large round bodies. Many of the distorted forms and large round bodies were coarsely granular. Strain 9S inoculated on AJA medium containing 10 per cent. serum and 50 units/ml penicillin when examined on the 2nd day of incubation had a moist confluent growth, a number of colonies growing into the agar and a few surface colonies which were not unlike revertant L type colonies. Microscopically, all these colonies showed the same cellular elements in approximately

similar proportions. L elements (various sizes) constituted approximately 50-60 per cent. of the total elements. The transitional filamentous forms made up 30-40 per cent. and bacillary forms constituted about 10 per cent. The filaments were extremely long, unlike the shorter filaments of 9R. These findings suggested that all the colony types found on the serum-penicillin AJA medium were heteromorphic L colonies. When the plates were examined on the 7th day of incubation a confluent growth was present with a number of colonies growing into the agar. The microscopical composition of these colonies (i.e. both confluent and deep colonies) consisted predominantly of transitional filamentous forms. There were large numbers of bacillary forms in both the surface and deep colonies particularly in the surface colonies where they constituted almost 30-40 per cent. of the total cellular elements. There were considerably more L elements in the deep colonies, than in the surface growth where they did not constitute more than 20 per cent. of the total elements. None of the L elements was demonstrable by Gram's staining method. The most predominant L elements were the small and medium spherical elements but large numbers of tiny spherical elements were also present.

INDUCTION OF L FORMS IN LABORATORY STRAINS (9S and 9R) WITH IMMUNE SERUM AND COMPLEMENT

(a) Induction of L forms with immune sera

Immune serum prepared in rabbits against Salm. gallinarum strain 9S was used in these experiments. The immune serum used had a titre of 1/2560 as determined by the tube agglutination test. Concentrated 24-hour PW cultures of strains 9S and 9R were plated out on AJA medium in which immune serum had been incorporated to give a final concentration of 10 per cent. AJA medium containing the same concentration of non-immune rabbit serum served as a control. Inoculated plates were incubated at 30°C and examined at the 3rd and 10th days of incubation. Cultures of 9S bacillary forms inoculated on to AJA medium containing immune serum and examined on the 3rd day of incubation showed a confluent, greyish-white, opaque, mucoid growth consisting of giant bacillary rods and large numbers of swollen filamentous forms. When the plates were re-examined on the 10th day of incubation the colonial morphology remained unaltered but changes in the microscopical composition of the colony were observed. The growth was now comprised of short and medium sized swollen rods and coccobacillary forms. However, small numbers of long rods and short filaments were also present. No L elements were encountered in either the 3 or

10 day cultures. On medium containing non-immune serum a confluent, greyish-white, mucoid growth was also present by the 3rd day of incubation and this consisted predominantly of medium and short turgid rods. Few to moderate numbers of coccobacillary and filamentous forms were also present. With strain 9R no significant differences were noted between growth obtained on media containing immune and non-immune serum. The 3- and 10-day incubated growth consisted predominantly of very long rods and to a lesser extent short rods. In addition, the 10-day incubated cultures had many coccobacillary forms.

(b) Induction of L forms with immune serum and complement.

In these experiments immune serum was not incorporated into the induction media, but was added to PW cultures of the bacillary forms in the presence of normal guinea-pig complement. Three immune sera having titres of 1/320, 1/640 and 1/1280 respectively were used. Overnight PW cultures of 9S containing approximately 1.5×10^9 cells/ml were mixed in 1.0 ml. amounts with 1.0 ml. of each immune serum. To each was added 1.0 ml reconstituted normal guinea-pig complement. The mixture of bacilli, immune serum and complement was allowed to stand at 37°C for 30 minutes before plating it out on AJA medium containing 10 per cent. inactivated horse serum. The inoculated plates were incubated at 30°C and examined at

intervals.

Plates inoculated with bacilli exposed to the immune serum with titre 1/320 produced an opaque, mucoid growth within 48 hours incubation. The main cytological elements in this growth were tiny and medium sized rods. There were large numbers of long filaments many of which were showing saccular swellings and spherical enlargements along their length. When examined on the 8th day there were now almost equal proportions of pleomorphic rods, transitional filamentous forms and coccobacillary forms. A small number of medium sized and large spherical elements were also present.

Bacilli allowed to react with higher titred immune serum (1/640) also produced an opaque, confluent moist growth within 48 hours. This 48-hour growth consisted mostly of long and medium sized rods, short filaments and occasional L elements, but the same growth examined on the 8th day of incubation showed short filaments, club-shaped forms, yeast-like cells, coccal forms and large numbers of coccobacilli. Bacilli exposed to the immune serum with a titre of 1/1280 produced an opaque, moist, confluent growth as well as structures that within 48 hours resembled revertant type L colonies not unlike those shown in plate 29. Microscopically both the confluent and revertant type of growth consisted mainly of bacillary rods and large numbers of filaments. There

were also small numbers of L elements (spherical elements of various sizes). The growth became more mucoid and had turned yellowish brown by the 8th day of incubation. When the surface growth was removed a number of colonies were seen growing into the agar. These colonies were similar to those shown in plate 23. Microscopically the confluent growth was essentially similar to the 48-hour growth. Colonies growing into the agar had an admixture of bacilli, transitional forms and a few L elements, suggesting that it was probably a reverting heteromorphic growth or more likely poorly transformed heteromorphic L colonies.

INDUCTION OF L FORMS ON AJA MEDIUM CONTAINING HIGH CONCENTRATION OF SUCROSE

These experiments were done in conjunction with experiments described in pages 274 to 281 and therefore the AJA medium containing 10 per cent. serum and usual amount of sucrose which served as controls in those experiments were taken as controls for these observations. Overnight PW cultures of strains 9S and 9R were plated out on AJA (E) medium (30 per cent. sucrose), containing 10 per cent. serum. Inoculated plates were incubated at 30°C. AJA(E) medium inoculated with bacillary forms of 9S produced a thin film of translucent, confluent growth within 24 hours. This growth became more opaque and profuse by the 4th day of incubation and in some parts of the plate there were colonies growing into the medium.

Gram's staining of the 4-day confluent growth showed predominantly pleomorphic bacillary forms many of which were swollen. A few small and medium sized spherical elements and occasional filamentous forms were present. There were also small amounts of pink-staining nondescript background material (suggesting the presence of other L elements which had been damaged by the Gram's staining technique). By means of Dienes' technique agar blocks bearing the confluent growth were stained and found to show the same elements revealed by Gram's method except that no spherical elements revealed by Gram's method were seen. However, when agar blocks bearing confluent growth and colonies growing into the agar were stained it showed both bacillary forms and small numbers of L elements in clusters. The apparent difference in the microscopical picture of the confluent growth as revealed by Dienes' and Gram's staining techniques was probably due to some of the colonies growing into the medium being picked up on the nichrome wire loop when making smears of the confluent growth for staining by Gram's method. With strain 9R the transformative changes were similar to those described for strain 9S. There was, however, no growth of colonies into the medium but the 4-day confluent growth contained, in addition to the bacillary elements, small numbers of L elements.

INDUCTION OF L FORMS IN FIELD STRAINS

STRAIN 37/74.

1. Induction of L forms with penicillin. Four-and-a-half-hour incubated PW cultures of strain 37/74 were plated out on the following media:

(a) AJA medium with no serum but containing 200 units/ml of penicillin.

(b) AJA medium containing 10 per cent. horse serum and 200 units/ml penicillin.

(c) AJA medium containing 20 per cent. horse serum and 200 units/ml penicillin.

(d) AJA medium containing no serum or penicillin.

(e) AJA medium containing 10 per cent. serum.

All inoculated plates were incubated anaerobically at 37°C and were examined once only at the 5th day.

On AJA medium containing only 200 units/ml of penicillin a heteromorphic L growth was produced which consisted predominantly of L elements and to a lesser extent of transitional rod forms. A small number of coccobacillary forms and tiny spherical elements were present. On AJA medium containing 10 or 20 per cent. serum and 200 units/ml penicillin, L colonies grew within the depths of the medium in addition to a patchy granular L growth on the surface. These L colonies consisted predominantly of L elements. On both the control AJA media the 5-day growth was bacillary

in appearance and consisted mainly of long and medium sized swollen rods and to a lesser extent, short and coccobacillary forms. A small number of non-bacillary elements was also present.consisting of intensely stained medium sized, small and tiny spherical elements some of which were slightly stained similar to those shown in plate 38.

2. Induction of L forms with glycine.

Overnight concentrated PW cultures of strain 37/74 were plated out on 10 per cent. serum AJA medium containing 1.5 and 3.0 per cent. glycine. The control AJA medium contained only 10 per cent. horse serum. The inoculated plates were incubated at 30°C. With 1.5 per cent. glycine in the induction medium this strain produced within four days greyish-white classical heteromorphic L colonies varying in size from less than 0.5 mm to 3.0 mm in diameter. All had a dark central core surrounded by a wide comparatively lighter periphery similar to the colonies shown in plate 39. This heteromorphic L colony consisted predominantly of lightly stained large round pale-staining bodies and large numbers of spherical elements of various sizes, the small and medium sized ones being most abundant. Also present in moderate to large numbers were irregularly shaped forms (probably distorted forms of the larger L elements viz. large round pale-staining bodies and large

spherical elements). Many medium sized turgid rods were present. Many of the distorted large bodies were coarsely granular and, in some, tiny bacilli were present. In some instances there was the impression that clusters of rods had arisen from some of these large bodies similar to those shown in plate 40. A notable feature was the virtual absence pf filaments. When these plates were examined on the 17th day the colonial appearance of the growth on these plates remained unchanged (see plate 39) except that a number of closely situated colonies had coalesced. Preparations of these 17-day colonies stained by Dienes' method showed them to consist predominantly of small spherical elements. A few medium sized spherical elements were present but no rods or filaments were seen. Likewise, large spherical elements and large round pale-staining bodies were also absent.

On media containing 3 per cent. glycine a patchy granular L type growth was present by the 4th day of incubation. Smears of this stained by Gram's method showed it to consist of masses of nondescript pink staining material and a few short turgid rods and coccobacillary forms. However, when these colonies were stained by Dienes' method they were shown to be composed mainly of large round pale-staining bodies and a few large spherical elements. No filaments or small or tiny spherical elements were present. A few unstained bacillary forms were encountered. By the 14th day of

incubation there appeared to be no further colony development and Gram's smears did not reveal any recognisable cellular elements. Dienes' stained preparations of this growth revealed only a few unstained, medium sized, spherical bodies, indicating that the growth had died out.

On control AJA medium containing only 10 per cent. serum. a well deveoped, dirty white, confluent bacillary type of growth was present. This growth, however, also had a slightly granular appearance and a Gram's smear (see plate 38) revealed large numbers of swollen long, medium and short rods and small numbers of turgid coccobacillary forms. Filaments were completely absent. About 10-15 per cent. of the total cells in this Gram's smear consisted of non-bacillary elements, viz. medium, small and tiny spherical bodies. Small amounts of light pink nondescript material was also present. On the 14th day of incubation, the growth had become more profuse and opaque; Gram's and Dienes' stained preparations showed it to consist of large numbers of tiny coccobacilli, often indistinguishable from the tiny spherical forms, and large numbers of short bi-polar stained rods. Filaments and L elements were completely absent.

STRAIN 846/71

1. Induction of L forms with penicillin.

Four-and-a-half-hour incubated concentrated PW cultures of strain 846/71 were plated out on

(a) AJA medium containing no penicillin but with 20 per cent. horse serum.

(b) AJA medium containing 200 units/ml of penicillin.

(c) AJA medium containing 20 per cent. horse serum and 200 units/ml of penicillin.

Inoculated plates were incubated anaerobically at 37°C and examined on the 5th day of incubation.

On both the media containing penicillin the growth was extremely poor and consisted of a very faint, confluent, granular L type growth. As the agar was soft some of the inoculum had seeped to the bottom of the plate and produced on the bottom face of the medium a growth which was considerably more profuse. The surface growth obtained on AJA medium containing both penicillin and serum was marginally more profuse than in the corresponding AJA medium containing only penicillin, but microscopically no significant differences were apparent between these media. Gram's smears of the confluent granular growth showed no bacillary or transitional forms but consisted of a mass of undifferentiated light pink staining material and a small number of what appeared to be considerably damaged, but still recognisable, small and medium sized spherical elements. The microscopic appearance of this growth in Gram's smears was similar to that of another strain shown in plate 42 but Dienes' staining of this 5-day growth showed it to consist almost entirely of

L elements of varying sizes and large numbers of very coarse blue staining granules. No rods or filamentous forms were present. The control medium produced a greyish white, moist and confluent growth. Gram's smears of this growth showed it to consist predominantly of short turgid rods, with smaller numbers of long and medium sized rods and coccobacillary forms. In addition to these cellular elements a small amount of light pink staining nondescript material was present. As well as the bacillary elements, moderate numbers of L elements (mainly medium sized and small spherical elements) and a small number of coarse blue staining granules were seen when Dienes' staining technique was used. No filaments or transitional forms were present in either Dienes' stained colonies or Gram's smears of this 5-day growth.

In a separate experiment L growth was obtained by plating out 24-hour concentrated PW cultures of this strain on AJA medium containing 10 per cent. serum and 200 units/ml penicillin. Control plates of AJA medium (containing no penicillin) with and without serum were seeded with the same cultures, incubated at 30°C and examined once only on the 9th day of incubation. On the AJA medium containing penicillin, a patchy granular L type growth was present. This was moderately well developed in some areas of the plate and microscopically consisted entirely of L elements and granules with no

filaments or bacillary forms present. In the control plates an opaque, greyish white, moist, confluent growth was produced and the microscopical findings were similar to those with 20 per cent. serum AJA medium described in the earlier experiment. There was, however, considerably fewer L elements on AJA medium containing no serum. Filaments were not encountered in either control medium.

2. Induction of L forms with glycine

Concentrated 24-hour PW cultures of strain 846/71 were plated out on the following media:

(a) AJA medium containing no glycine or serum

(b) AJA medium containing only 10 per cent. horse serum

(c) AJA medium containing 1.5 per cent. glycine

(d) AJA medium containing 3.0 per cent. glycine

(e) AJA medium containing 10 per cent. horse serum and 1.5 per cent. glycine

(f) AJA medium containing 10 per cent. horse serum and 3.0 per cent. glycine.

All inoculated plates were incubated at 30° and examined on the 9th day of incubation.

On the control medium without glycine or serum, an opaque, confluent, greyish-white growth was present. Gram's smears of this revealed predominantly medium sized rods and, to a lesser extent, short rods. A few coccobacillary and coccal forms were also present. There was, however, a noticeable absence of filaments.

Dienes' staining of this same growth revealed moderate numbers of small spherical elements in addition to the other cells seen with Gram's stain.

On the medium with serum a moist, dirty white, confluent growth was present in which Gram's smears showed many short and medium sized rods and coccobacillary forms. Appreciable numbers of medium sized, small and tiny spherical elements were also present. Dienes' staining showed these L elements to constitute at least 15-20 per cent. of the total cellular elements.

On the medium containing 1.5 per cent. glycine a slimy, greyish-white confluent growth developed, the chief cellular constituents of which were (in order of abundance) short and medium sized rods, small spherical L elements, coccobacillary forms, tiny and large spherical L elements and large round pale-staining bodies. A small number of short filaments was also present. On medium containing 3.0 per cent. glycine a hardly visible growth was present and microscopically (both by Dienes' and Gram's staining techniques) no recognisable elements were seen.

On the medium with serum and 1.5 per cent. glycine a greyish-white, slimy growth was produced. Gram's smears showed this to consist predominantly of medium sized stout rods and pink staining nondescript material. A few short rods, coccobacillary forms and short filaments were also present. Dienes' stained

preparations of the same growth showed, in addition, large numbers of small and tiny spherical L elements with a much small number of large spherical elements and large round pale-staining bodies containing granules.

On the medium with serum and 3.0 per cent. glycine a very translucent, confluent, granular growth was present. The growth on this plate was more profuse than that produced on the medium with 3.0 per cent. glycine but no serum. Microscopically only L elements (predominantly large and medium sized spherical elements) and occasionally short filaments were present.

In a subsequent experiment 4 day cultures were examined. On the medium containing 10 per cent. serum and 1.5 per cent. glycine the 4-day growth consisted of not only a slimy, confluent growth but also many classical L type colonies. These L colonies, when stained by Gram's method, showed no recognisable L elements but a light pink staining nondescript material. Dienes' stained preparations showed these colonies to be composed almost entirely of L elements of all sizes, large round, pale staining bodies, distorted and irregularly shaped L elements and occasional transitional forms. The confluent growth presented an almost identical picture except that there were a few more transitional forms. The same growth when examined on the 17th day of incubation showed large numbers of long and medium sized rods in addition to the L elements.

On AJA medium containing 10 per cent. serum and 3.0 per cent. glycine a well developed patchy granular L type growth was present by the 4th day of incubation consisting entirely of L elements (mostly large spherical elements, large round, pale-staining bodies and medium sized spherical elements). Granules, small and tiny spherical elements were not observed. Filaments and rods were absent.

The control medium containing only 10 per cent. serum produced a creamy-white, moist, confluent growth by the 4th day of incubation. Microscopically the principal elements were short rods and coccobacilli but about 10-20 per cent. of the total elements were composed of small and tiny spherical elements. When this growth was examined on the 22nd day only a few tiny spherical elements were present.

3. Induction of L forms with penicillin and an increased amount of sucrose in the induction medium.

These experiments were conducted in conjunction with the experiments described on pages 299-300 and therefore no separate controls were included. Concentrated 24-hour PW cultures of strain 846/71 were plated out on AJA (E) medium (i.e. AJA medium containing 30 per cent. sucrose instead of the usual 15 per cent.) and 200 units/ml penicillin. Inoculated plates were incubated at 30°C and examined once only on the 9th day. There was only

a scanty patchy granular L type of growth on this medium even after 9 days' incubation. Microscopically no bacillary or transitional forms were present, the growth being composed only of L elements.

4. Induction of L forms with immune serum

Twenty-four hour concentrated PW cultures of strain 846/71 were plated out on the following media:-

(a) AJA medium containing no serum

(b) AJA medium containing 10 per cent. inactivated non-immune rabbit serum

(c) AJA medium containing 10 per cent. inactivated immune serum with a titre of 1/2560.

The inoculated plates were incubated at 30°C and were examined on the 3rd and 10th days of incubation. On AJA medium containing no serum, an opaque greyish white confluent bacillary type of growth was produced by the 3rd day. Gram's smears of this showed it to consist mainly of turgid coccobacillary forms and short rods and to a lesser extent of medium sized rods. Small to moderate numbers of small and tiny spherical elements were also present. Occasional long rods/ short filaments were sometimes observed. In addition to the above cellular elements small amounts of pink staining nondescript material were present. Dienes' stained preparation of this 3-day growth showed not only the same bacillary elements but also larger number of

small spherical forms and medium-sized as well as large spherical elements. The latter two types comprised about 10 per cent. of the total cellular elements. A few large round, pale-staining bodies and occasional transitional forms were also seen. When the growth was examined on the 10th day of incubation there were proportionately more bacillary forms and tiny spherical elements. The larger L elements (large spherical elements and large round, pale-staining bodies) were almost completely absent but the small spherical elements were still present in appreciable numbers.

On AJA medium containing 10 per cent. non-immune rabbit serum a dirty greyish-white thick confluent growth was present on the 3rd day and its microscopical composition was similar to that of the 3-day growth obtained on AJA medium containing no serum, the only notable difference being a slightly higher number of large L elements and transitional forms. The 10-day growth microscopically also resembled the corresponding growth obtained on AJA medium containing no serum. There were, however, slightly more medium sized spherical L elements on the AJA medium containing non-immune serum.

On AJA medium containing 10 per cent. immune rabbit serum a greyish-white, opaque growth was produced by the 3rd day of incubation. The microscopic appearance of this 3-day growth was not unlike that produced on AJA medium

containing non-immune serum, except that there were considerably more transitional rod and filamentous forms. The 10-day growth contained a larger number of coccobacillary forms and short rods but no filaments were present. There was, however, a substantial reduction in the number of L forms in the 10-day growth. There appeared to be less L elements in the 10-day growth produced on AJA medium containing immune serum than in the corresponding growth obtained on AJA medium containing non-immune serum.

5. Propagation of glycine-induced L forms.

a 9-day incubated slimy growth obtained on AJA medium containing 10 per cent. horse serum and 1.5 per cent. glycine (see pages 299-300 for microscopic composition of this growth) was subcultured on the following media:-

(a) AJA medium containing no glycine, penicillin or serum

(b) AJA medium containing 10 per cent. horse serum

(c) AJA medium containing 10 per cent. horse serum and 1.5 per cent. glycine

(d) AJA medium containing 10 per cent. horse serum and 3.0 per cent. glycine

(e) AJA$^{(E)}$ medium containing 10 per cent. serum and 200 units/ml penicillin.

Inoculated plates were incubated at 30^{o}C and examined on the 3rd and 9th days of incubation.

On AJA medium alone a thick greyish white bacillary type of growth was present by the 3rd day. The chief cellular elements found in this growth were medium sized rods many of which were swollen. Many long rods, coccobacillary forms and transitional rod forms were also present along with small numbers of spherical elements and large round, pale-staining bodies. By the 9th day this growth showed mainly swollen, coccobacillary forms. There was a considerable reduction of L elements in the 9-day growth compared with the 3-day growth. On AJA medium containing serum a growth similar in appearance to that produced on the medium without serum was present on the 3rd day of incubation. The microscopic composition of this growth was predominantly short turgid rods, with a few medium-sized and long rods and occasional short filaments. There were large numbers of L elements of various sizes but small and medium-sized spherical elements were the most frequently occurring types. Some large round, pale-staining bodies were also seen. The 9-day growth consisted of the same elements but the L forms were in small clusters. On serum-AJA medium with 1.5 per cent. glycine, the 3-day growth was greyish white and very slimy consisting microscopically of extremely tiny rods and small coccobacillary forms. There were also large numbers of tiny spherical elements and few to moderate numbers of small and medium sized spherical elements. No filaments, large spherical elements

or large round pale-staining bodies were seen. By the 9th day the growth had become more slimy and was rust coloured. This growth stained by Gram's method showed a mass of pink staining background material in which a variety of bacillary elements were present viz. coccobacillary forms, medium-sized and long rods. Moderate numbers of small and tiny spherical elements were also present as well as rather fewer medium-sized spherical elements. Dienes' staining of this growth could not be assessed satisfactorily due to the presence of yellow coloured crystalline material which covered some of the cellular elements.

On serum-AJA medium containing 3.0 per cent. glycine a slightly opaque, granular L type growth was present by the 3rd day of incubation. Gram's smears of this showed it to be completely devoid of any bacillary elements but some damaged, but still recognisable shrunken spherical elements could be made out. Dienes' stained preparations of this 3-day growth showed it to consist predominantly of the large spherical elements and large round pale-staining bodies. A few medium sized and small spherical bodies were also present. A number of the larger spherical elements and large round pale-staining bodies were distorted. Occasional grossly distorted transitional forms were encountered. The 9-day growth was similar to the 3-day growth in as far as its microscopical composition was concerned. On

AJA (E)medium with serum and penicillin the findings were almost identical to those described for the growth obtained on the medium with 10 per cent. serum and 3.0 per cent. glycine.

6. Effect of various pH values of the induction medium on L transformation.

Eighteen- to twenty-four-hour concentrated PW cultures of Strain 846/71 were plated out on AJA medium containing 10 per cent. horse serum and 100 units/ml penicillin. The pH values of the AJA medium had been previously adjusted to pH 6.7, 7.2 and 8.1. A control AJA medium (containing no penicillin or serum) with pH 7.2 was also inoculated. Inoculated plates were incubated at 30°C and examined once only on the 3rd day of incubation. On AJA medium at pH 6.7 a well developed patchy granular L type growth was present when the plates were examined on the 3rd day of incubation. Gram's smears of this growth showed it to be completely devoid of any bacillary or transitional elements. While Dienes' stained preparations consisted entirely of L elements, the most predominant being large and medium sized spherical bodies although many large round, pale staining elements and few to moderate numbers of small and tiny spherical elements were also present. Many of the L elements, particularly the large spherical elements and large round, pale-staining bodies were granular.

On AJA medium at pH 7.2 a well developed patchy granular L type growth was present by the 3rd day of incubation. This was more profuse than the 3-day growth obtained on AJA medium at pH 6.7 but its microscopical composition was almost identical to that obtained on AJA medium at pH 6.7.

On AJA medium at pH 8.1 the growth after 3 days consisted of L colonies with a classical morphology. A number of neighbouring L colonies were found to have coalesced. These L colonies consisted predominantly of L elements, but a few short filaments were also present. On control AJA medium at pH 7.2 (i.e. containing neither serum nor penicillin) a confluent bacillary type of growth was present by the 3rd day of incubation. Microscopically the most predominant forms found in this growth were medium sized and short rods. The most unusual finding in this 3-day bacillary type of growth on the control AJA medium was the presence of large numbers of non-bacillary forms which made up almost 30 per cent. of the total cellular elements. These non-bacillary cellular elements consisted mostly of tiny and small spherical elements.

STRAIN 154/71

1. Ability of penicillin-treated broth cultures to produce L growth on AJA medium.

To a 3-hour nutrient broth culture of strain

154/71 incubated at 37°C, penicillin was added to give a final concentration of 40,000 units/ml penicillin of the broth culture. After the addition of penicillin the culture was incubated at 37°C for a further period of 20-25 minutes before it was plated out on the following AJA media:

(a) AJA medium containing no penicillin or serum,

(b) AJA medium containing 10 per cent. horse serum and no penicillin,

(c) AJA medium containing 20 per cent. horse serum and no penicillin.

After inoculation, plates were incubated at 30°C. In neither of the AJA media containing serum was growth of any kind present, up to the 13th day of incubation. Of the two inoculated plates containing only plain AJA medium (i.e. containing neither serum nor penicillin) one gave no growth over the 13 days of incubation, but the 2nd plate showed a confluent bacillary growth on the 5th day of incubation. In addition to the confluent growth, a few classical heteromorphic L colonies were present. A number of colonies were also developing within the depths of the agar. The colonies growing within the depths of the agar as well as the surface classical heteromorphic L colonies were examined microscopically. The chief cellular elements found in both these colony types were L elements of various sizes and large round, pale-staining bodies. There appeared to be a

preponderance of the small and tiny spherical L elements. Many of the large spherical elements and large round, pale-staining bodies were coarsely granular. There were many distorted forms of the large round bodies. Also occurring in small to moderate numbers were transitional filamentous forms. A few long rods were occasionally seen.

2. Induction of L forms with glycine.

Twenty-four-hour incubated concentrated PW cultures of strain 154/71 of Salm. gallinarum were inoculated on the following media:

(a) AJA medium containing 10 per cent. horse serum (control),

(b) AJA medium containing 10 per cent. horse serum and 1.5 per cent. glycine,

(c) AJA medium containing 10 per cent. horse serum and 3.0 per cent. glycine.

Inoculated plates were incubated at 30°C.

When the control AJA medium was examined on the 4th day of incubation, the plates were covered with a greyish-white, slightly opaque, confluent growth. Microscopically this growth appeared to be very pleomorphic (long, medium-sized and short rods, coccobacilli and short filaments were present). Apart from these bacillary forms there were also small numbers of tiny and small spherical elements. On AJA medium containing 1.5 per cent. glycine and 10 per cent.

serum an opaque, moist, confluent growth was present by the 4th day of incubation. Gram's smears of this growth showed it to consist of large numbers of small swollen rods which terminated in pointed ends, and there were also large amounts of pink staining non-descript material. A few medium-sized rods and a small number of tiny spherical elements were also present. No filaments were seen. When the same growth was examined by Dienes' staining technique, it appeared that almost 50 per cent. of the cellular forms were non-bacillary elements, consisting mainly of tiny, small and medium sized spherical elements and a few large round, pale-staining bodies. By the 12th day the growth on this medium containing glycine had become slimy and more opaque. Gram's smears showed masses of pink staining nondescript material and against this background was a number of well stained tiny, small and medium sized spherical elements. Rods and filaments were virtually absent except for occasional giant bacillary forms or grossly enlarged yeast-like bodies (both of which could be considered as transitional forms). Using Dienes' staining technique, this 12-day growth consisted almost entirely of transformed elements consisting mainly of small and tiny spherical elements and large numbers of blue staining granules. Other elements found in small numbers were large and medium sized spherical elements, large round, pale-staining bodies and occasional transitional

forms.

On AJA medium containing 10 per cent. serum and 3 per cent. glycine, growth was extremely scanty even after 4 days, and consisted of a patchy, granular L type growth. This growth, when stained by Gram's method, showed no recognisable cellular elements and the whole smear appeared to consist of pink staining nondescript material, but Dienes' stained preparations of the same growth showed it to consist entirely of L elements, almost 80 per cent. of them consisting of large round pale-staining bodies, with smaller numbers of large, medium and small spherical elements.

3. Induction of L forms on medium containing high concentrations of sucrose.

Twenty-four-hour concentrated PW cultures of strain 154/71 of Salm. gallinarum were plated out on AJA medium containing the usual strength of sucrose (i.e. 15 per cent.) and 10 per cent. horse serum and on AJA(E)medium (i.e. AJA medium with 30 per cent. sucrose) containing 10 per cent. horse serum. Inoculated plates were incubated at 30°C and examined once only on the 4th day. On the AJA medium containing the usual concentration of sucrose a confluent, greyish-white growth was present which consisted of medium-sized rods and, to a lesser extent, long and short rods; coccobacillary and tiny spherical forms were present in small numbers. Small spherical elements were occasionally seen. On

the corresponding AJA(E)medium in addition to the confluent growth a number of colonies were found growing into the agar under the confluent growth. Microscopically the confluent growth consisted in the main of coccobacillary forms and short rods but almost 30 per cent. of the total cellular elements were composed of tiny spherical forms. In addition there were small numbers of large, medium and small spherical elements and a few large round, pale-staining bodies.

4. Effect of the pH of induction media on L transformation

Eighteen- to twenty-hour concentrated PW cultures of strain 154/71 were inoculated on AJA medium containing 10 per cent. horse serum and 100 units/ml penicillin. The pH of the AJA medium had been adjusted previously to 6.7, 7.2 and 8.1. The control AJA medium had no serum or penicillin and its pH was adjusted to 7.2. Inoculated plates were incubated at 30°C.

On the control AJA medium a slightly opaque, confluent growth was present by the 3rd day of incubation. This consisted predominantly of small rods, coccobacillary and tiny spherical forms, a smaller number of medium-sized rods were also present but no long rods or filaments were seen.

On AJA medium at pH 6.7 a faint, confluent, granular L type growth was present on day 3 of incubation. Staining of this growth by Dienes' method showed it to

consist predominantly of large round, pale-staining bodies and large spherical elements. There were only a few medium-sized spherical elements. No small or tiny spherical elements were seen, neither were there any bacillary or transitional forms. Many of the larger forms appeared to be slightly distorted. The growth on this medium was considerably more profuse by the 7th day of incubation and Gram's smears showed only pink staining nondescript material.

On AJA medium at pH 7.2 a thin, confluent granular L type growth was present by the 3rd day of incubation. Microscopically the predominant elements were the large and medium-sized spherical elements, the large round,, pale-staining bodies occurred in moderate numbers and a few small spherical elements were also present, but no tiny spherical elements were seen, nor were bacillary or transitional elements present. The growth had become more opaque by the 7th day of incubation but microscopically there was evidence of mass reversion. In this 7-day growth, there were large numbers of bacillary forms (Almost 50-60 per cent. of the total elements). Most of the bacillary forms were long and medium sized rods. A few short filaments were also present. Present in moderate to large numbers were L elements of all sizes (predominantly small and tiny spherical elements) and some coarse blue staining granules.

On AJA medium at pH 8.1 by the 3rd day of incubation

there were many classical L type colonies present (similar to those shown in plate 39) and microscopically these colonies consisted mainly of L elements of various sizes (large, medium, small and tiny spherical elements), although there were considerable numbers of transitional rod and filamentous forms, while large round, pale-staining bodies were few in number. There appeared to be very little distortion of the L elements, many of which were also present in Gram's smears of this 3-day growth. By the 7th day some of the L colonies had transformed to a morphology more like the revertant type (see plate 29) and in addition classical L colonies and confluent growth were present. The L colonies with a classical morphology now consisted principally of small and tiny spherical elements with large numbers of large and medium-sized spherical elements. Also present in large numbers were transitional filamentous forms. A few large round, pale-staining bpdies and moderate numbers of blue staining granules were present. No rod forms were seen.

STRAIN 131/71

1. Induction of L forms with penicillin.

Twenty-four-hour concentrated PW cultures of strain 131/71 of Salm. gallinarum were plated out on AJA medium containing 10 per cent. horse serum and 50 units/ml penicillin and on similar AJA medium not

containing penicillin. The inoculated plates were incubated at 30°C and were examined on the 3rd and 7th days of incubation. On AJA medium containing penicillin a well developed patchy granular L type growth was produced by the 3rd day. When this growth was stained by Gram's method, no recognisable cellular elements were present except for pink staining nondescript material. By Dienes' staining technique this growth was seen to consist predominantly of small and medium-sized bodies and large spherical elements were also present but no transitional or rod forms were seen. When the plates were examined on the 7th day the growth had become more opaque and confluent and a number of colonies were growing into the agar beneath the confluent growth. The 7-day confluent growth stained by Gram's method revealed many transitional filamentous forms as well as a number of well-stained medium-sized, small and tiny spherical elements and moderate amounts of nondescript pink staining material. A few short filaments and occasional rod forms were also encountered. This growth stained by Dienes' method showed that almost 70 per cent. of the total cellular forms consisted of L elements of all sizes. The medium and small spherical elements were the major L elements though there were significant numbers of large and tiny spherical L elements. Only a small number of large, round, pale-staining L bodies were present. The bulk of the remaining elements were the

filamentous forms, most of them with large saccular dilatations (transitional forms). Rod forms were rare.

On control AJA medium not containing penicillin, L forms of the larger varieties were not observed in either of the 3- and 7-day incubated growths. The 3-day growth consisted predominantly of long and medium-sized swollen rods and to a lesser extent short stout rods and coccobacillary forms, whereas the 7-day growth showed more swollen coccobacillary forms and fewer swollen long and medium-sized rods. Some tiny spherical forms were also present.

2. Induction of L forms with glycine.

Twenty-four-hour concentrated PW cultures of strain 131/71 were plated out on the following AJA media:

(a) AJA medium containing no serum (control 1),

(b) AJA medium containing 10 per cent. serum (control 2),

(c) AJA medium containing 10 per cent. serum and 3 per cent. glycine,

(d) AJA medium containing 3 per cent. glycine,

(e) AJA medium containing 1.5 per cent. glycine.

Inoculated plates were incubated at 30°C and were examined on the 4th and 14th days of incubation.

On AJA medium containing no serum or glycine, a moist, confluent bacillary type growth was present by the

4th day of incubation, consisting mainly of long and medium sized slender rods and to a lesser extent small rods and coccobacillary forms. This growth, when examined 10 days later, showed the same elements but most of them were swollen. No L elements were present in either the 4-day or 14-day growths.

On AJA medium containing only 10 per cent. serum a greyish-white, slightly opaque, confluent growth was present by the 4th day consisting predominantly of very large swollen coccobacillary forms and stout medium-sized rods. Also present were giant bacillary and coccal forms. On the 14th day the growth consisted of coccobacillary forms which were not so swollen and many short stout rods. No large L elements were present in either the 4-day of the 14-day growths.

On AJA medium containing 10 per cent. serum and 3 per cent. glycine a faint patchy granular L type growth was present when the plates were examined at day 4 of incubation. This growth contained no bacillary or transitional elements and was made up of L elements (many large and medium-sized forms) and some coccal elements. When the growth was examined on the 14th day of incubation it had not increased significantly in amount. Microscopically there were no signs of reversion, the principal elements found being large and medium-sized spherical elements and many distorted forms of the large, round, pale-staining bodies; there were

also large numbers of very coarse blue staining granules.

On AJA medium containing 3 per cent. glycine, the 4-day incubated growth was extremely meagre, being very much less than in the corresponding AJA medium containing 3 per cent. glycine and 10 per cent. serum. The chief cellular elements were very large and medium-sized spherical elements and large numbers of coarse blue granules. After a further period of 10 days' incubation there was no appreciable increase in the amount of growth on this medium. Gram's smears of this 14-day-old growth showed no transitional or bacillary elements but Dienes' staining showed many tiny coccobacillary forms stained intensely at the poles, and tiny and small spherical forms. The true identity of the tiny bipolar coccobacillary forms could not be established, but it did not appear that these were bacillary forms as they could not be stained by Gram's staining technique.

On AJA medium containing 1.5 per cent. glycine a rust coloured, moist, confluent growth was present when the plates were examined on the 4th day of incubation. Microscopically almost 70 per cent. of this 4-day growth was made up of L elements (spherical elements of various sizes and blue stained granules). There were many long and medium-sized rods and short and long filaments. Most of the rod forms ended in sharp points. Gram's smears of the 14-day growth showed the cultures now to consist of spherical elements

of all sizes many of which appeared to be damaged, along with vast amounts of granules of pink staining nondescript material and a few elongated yeast-like forms (transitional rod forms). No normal bacilli or filaments were present. Dienes' stained preparations showed, in addition, large round, pale-staining bodies many of which appeared to be distorted. There were also many large spherical elements that were not seen in Gram's smears. Tiny spherical forms were also present.

3. Induction of L forms on medium containing high concentrations of sucrose.

These experiments were carried out in conjunction with the preceding experiments and therefore the AJA medium control 1 (see page 318) served also as control for this experiment.

Twenty-four-hour concentrated PW cultures of strain 131/71 were plated out on AJA(E) medium. The inoculated plates were incubated at 30°C and were examined on the 4th and 14th days of incubation. On day 4 of incubation on the AJA(E) medium a slightly opaque, greyish-white confluent growth was present. Microscopically there was a mixture of swollen coccobacillary forms, short swollen rods, large numbers of spherical elements of various sizes. Some of the large, round, pale-staining bodies contained coarse dark blue granules which were also found extracellularly. When the growth was

examined microscopically on the 14th day of incubation it consisted of swollen coccobacillary forms, swollen short rods and a few tiny spherical forms.

STRAIN 784/71

1. Induction of L forms with glycine.

Twenty-hour concentrated PW cultures of strain 784/71 were inoculated on the following AJA media:

(a) AJA medium containing 10 per cent. horse serum,

(b) AJA medium containing 10 per cent. horse serum and 1.5 per cent. glycine,

(c) AJA medium containing 10 per cent. horse serum and 3.0 per cent. glycine.

Inoculated plates were incubated at 30°C.

On AJA medium containing 10 per cent. serum a slightly opaque, confluent growth was present by the 4th day of incubation and consisted predominantly of coccobacillary forms, and to a lesser extent short stout rods and medium-sized rods. A few long rods were also present. Present in small numbers were tiny, small and medium-sized spherical elements. These non-bacillary forms constituted about 5 per cent. of the total cellular elements. Filamentous forms were absent. When the growth was examined 10 days later, it consisted almost entirely of tiny coccobacilli hardly distinguishable from coccal forms. A few short and medium-sized rods were also present. Long rods, filaments and spherical elements were absent.

A moderately opaque, confluent L type growth was present on AJA medium containing 10 per cent. serum and 1.5 per cent. glycine by the 4th day of incubation. A number of compact colonies (not unlike those shown in plate 23) were found growing into the agar beneath this layer of confluent growth. The 4-day incubated confluent growth stained by Gram's method was seen to consist of many L elements of all sizes, and small amounts of pink staining nondescript material. Many of the L elements appeared slightly distorted in shape (see plate 41). There were no rods or filaments. With Dienes' staining the growth showed considerably more L elements and also substantial numbers of large, round, pale-staining bodies which were not revealed by Gram's staining technique. Many of the large round bodies were grossly distorted and assumed irregularly shaped forms.

On AJA medium containing 10 per cent. serum and 3.0 per cent. glycine only a feeble patchy granular L type growth was produced even after 4 days' incubation. Gram's smears of this growth showed the complete absence of recognisable cellular elements, but with Dienes' staining technique a few well stained, large spherical elements were seen. Most of the other L elements i.e. small and medium-sized spherical elements and large round, pale-staining bodies did not appear to be stained; there was, however, a complete absence

of bacillary rod forms and filaments. When the plates were re-examined on the 14th day of incubation no increase in the growth was noted. Microscopically the findings were almost identical to those found in the 4-day growth, indicating that the growth had not developed further beyond the 4th day of incubation.

2. Induction of L forms with penicillin.

Although controls were not included in the following experiments the findings are reported here to show the effects of penicillin in bringing about L transformation in strain 784/71. Concentrated 4½-hour PW cultures of this strain of Salm. gallinarum were inoculated on AJA medium containing 20 per cent. horse serum and 200 units/ml penicillin. The inoculated plates were incubated anaerobically at 37°C.

By the 4th day of incubation a well developed patchy granular L type growth was present and, in addition, a number of globular atypical L colonies had developed within the depths of the agar, similar to those as shown in plate 19. Microscopically both the patchy, granular L type growth and the atypical L colonies consisted of small and medium-sized spherical elements and to a lesser extent large spherical elements. There were also large numbers of coarse blue staining granules (observed only in Dienes' staining method). No bacillary rods and filamentous forms were seen. When the growth on these plates was examined on the 11th day of incubation, the

surface growth had become more opaque and confluent but no visible changes were observable in the atypical L colonies. Gram's smears of the 11-day confluent growth showed it to consist predominantly of well stained tiny and small spherical elements and moderate numbers of slender filaments, most of which were long. A few long and medium-sized rods were also present. When the growth was stained by Dienes' method it was observed that 80-85 per cent. of the total cells consisted of tiny, small and medium-sized spherical elements. Most of the medium-sized spherical elements were coarsely granular. There were also vast numbers of blue staining granules. Filamentous transitional forms constituted about 10 per cent. of the elements and the rest were bacillary rods.

3. Induction of L forms with immune serum.

Twenty-four-hour concentrated PW cultures of strain 784/71 of Salm. gallinarum were plated out on the following AJA media:

(a) AJA medium containing 10 per cent. immune rabbit serum (titre 1/2560),

(b) AJA medium containing 10 per cent. non-immune rabbit serum

(c) AJA medium containing no serum at all. Inoculated plates were incubated at 30° and were examined on the 3rd and 10th days of incubation.

On AJA medium containing immune serum a slightly opaque, greyish-white, confluent growth was produced by the 3rd day of incubation consisting of a mixture of long, medium-sized and short rods in almost equal proportions and small numbers of coccobacillary forms. No filaments were seen, but occasional small spherical elements were encountered. The 10-day growth consisted of essentially the same bacillary elements but most were greatly swollen and spherical elements were absent.

On AJA medium containing non-immune rabbit serum a similar greyish-white, confluent growth was produced which microscopically was almost identical to the 3-day growth produced on AJA medium containing immune serum. Likewise, the microscopical findings of the 10-day growth was not unlike that found in the corresponding culture on AJA medium containing immune serum.

On AJA medium containing no serum a greyish-white, confluent growth was present by the 3rd day of incubation. A most unusual microscopic finding in this growth was the exceptionally high numbers of the spherical elements present which constituted about 30 per cent. of the total cellular elements. The predominant nonbacillary forms were the small and tiny spherical elements. Large spherical elements and large round, pale-staining bodies were very few in number. About 60 per cent. of the remaining cells were bacillary rods and the rest were transitional forms. When the growth was examined on

the 10th day there was a complete absence of the nonbacillary elements. The chief elements found in this 10-day growth were medium-sized and short turgid rods and a few coccobacillary forms.

STRAIN 892/71

1. Induction of L forms with penicillin.

Eighteen-hour concentrated PW cultures of strain 892/71 were subcultured on AJA medium containing 10 per cent. horse serum and 50 units/ml penicillin and on AJA medium containing 10 per cent. horse serum. Inoculated plates were incubated at 30°C.

A patchy, granular L type growth was produced on the AJA medium containing penicillin by the 3rd day of incubation and when this growth was examined by Gram's method no recognisable cellular elements were present, but by Dienes' staining technique the growth was shown to consist entirely of L elements. The small and medium-sized spherical elements were the chief cellular forms, although there were substantial numbers of large spherical bodies. A few large round, pale-staining bodies were also present. By the 7th day of incubation the growth had become more opaque and there were also globular atypical L type colonies similar to those shown in plate 19 present in the depths of the agar. When the 7-day confluent growth was examined microscopically there were signs that it was beginning to revert; this 7-day growth consisted predominantly of small and

medium-sized spherical elements and in lesser numbers, large spherical forms and large round, pale-staining bodies. There were also a few long rods and transitional rod forms. On AJA medium containing no penicillin, a confluent bacillary type of growth was present by the 3rd day of incubation. Microscopically the growth appeared to consist predominantly of long, medium sized and short rods, and there were also very considerable numbers (constituting approximately 10-20 per cent. of the total cells present) of tiny, small and medium-sized spherical elements present. Although a few of these L forms could be recognised in Gram's smears, most of them were best seen in Dienes' preparations. By the 7th day the growth had become more opaque and was now made up mostly of short and medium-sized rods and coccobacillary forms. The L elements (tiny, small and medium-sized spherical bodies) still constituted about 10-20 per cent. of the total cellular elements.

2. Induction of L forms with glycine

Twenty-four-hour concentrated PW cultures of Strain 892/71 were inoculated on the following media:
(a) AJA medium containing 10 per cent. horse serum,
(b) AJA medium containing 10 per cent. horse serum and 1.5 per cent. glycine.
Inoculated plates were incubated at 30°.

On AJA medium containing only 10 per cent. serum an opaque, confluent growth was present by the 4th day of incubation. Microscopically this growth consisted mainly of long and medium-sized rods; short rods and coccobacillary forms were present in small numbers. There were large numbers of small and tiny spherical elements and a few medium-length filaments. On the medium containing glycine a confluent growth as well as discrete slimy colonies were found by the 4th day of incubation. When these colonies were stained by Gram's method they showed masses of pink staining nondescript material and many small and medium-sized damaged spherical elements (see plate 42). The same growth when examined by Dienes' staining technique, was seen to be made up of L elements ranging from tiny to large spherical elements (see plate 43). Transitional elements constituted less than 1.0 per cent. of the total cells.

3. Induction of L forms with glycine and penicillin.

Eighteen-hour concentrated PW cultures of 892/71 were plated out on the following media:

(a) AJA medium containing 10 per cent. horse serum,

(b) AJA medium containing 1.5 per cent. glycine,

(c) AJA medium containing 1.5 per cent. glycine, 10 per cent. horse serum and 50 units/ml penicillin,

(d) AJA medium containing 3.0 per cent. glycine, 10 per cent. horse serum and 50 units/ml penicillin.

On AJA medium containing only 10 per cent. serum both the macroscopic and microscopic findings were similar to those described on page 328 (see under induction of L forms with glycine). In both the 2-day and 7-day growths on this medium, the small and tiny spherical elements constituted between 10-20 per cent. of the total cellular elements.

On AJA medium containing only 1.5 per cent. glycine a very scanty, slightly opaque, confluent growth was present by the 2nd day of incubation. This growth became more opaque and moist by the 7th day and consisted of large numbers of medium-sized, small and tiny spherical elements. There were also moderate numbers of medium-sized and short rods and a number of very tiny rods. Filaments were rarely encountered.

On AJA medium containing 10 per cent. serum, 1.5 per cent. glycine and 50 units/ml penicillin, a very thin film of confluent, granular L type growth was present by the 2nd day of incubation. Gram's smears of this 48-hour growth showed only pink staining nondescript material but by Dienes' staining method this growth showed mainly medium-sized and, to a slightly lesser extent, large spherical elements. Many small spherical elements were also present. There was a complete absence of transitional or bacillary elements.

On AJA medium containing 10 per cent. serum, 3 per cent. glycine and 50 units/ml penicillin only the

faint beginnings of a scattered, patchy, granular L type growth were present when these plates were examined on the 2nd day of incubation. This growth became more opaque by the 7th day and microscopically consisted of medium-sized spherical elements. No rods or transitional forms were present.

4. Induction of L forms on AJA medium containing high concentrations of sucrose.

This experiment was carried out in conjunction with experiments on the induction of L forms with glycine (see page 328) and therefore the AJA medium containing 10 per cent. horse serum used in that experiment served as a control for this experiment. Twenty-four concentrated PW cultures of strain 892/72 were inoculated on AJA(E)medium containing 10 per cent. horse serum. Inoculated plates were incubated at 30°C and examined on the 4th day of incubation. The growth produced on this medium was similar to that produced on the control AJA medium except that several compact colonies were growing into the agar beneath the confluent growth. Microscopically the findings were also not unlike those described for the controlAJA medium, but there were considerably more L elements on the high sucrose medium. The L elements comprised between 30 and 40 per cent. of the total cells present.

5. Induction of L forms with immune serum alone.

Twenty-four-hour concentrated PW cultures of

strain 892/71 were inoculated into the following media:

(a) AJA medium containing no serum,

(b) AJA medium containing 10 per cent. non-immune rabbit serum,

(c) AJA medium containing 10 per cent. rabbit serum with a titre of 1/2560.

Inoculated plates were incubated at 30°C and were examined on the 3rd and 10th days of incubation.

On AJA medium containing no serum a greyish-white confluent growth was present by the 3rd day of incubation. The chief bacillary forms encountered in this 3-day growth were relatively slender medium, long and short rods and many coccobacillary forms. L elements constituted about 30 per cent. of the total cells present, the chief ones being the small and tiny spherical elements and a few medium-sized spherical elements. Many of these elements could be recognised on Gram's smears. No filaments were present. When the growth was examined on the 10th day of incubation it consisted mostly of tiny rods and coccobacillary forms, although there were significant numbers of long and medium-sized rods. Occasional filaments were also encountered. L elements now constituted less than 2 per cent. of the total cellular elements.

On AJA medium containing non-immune serum, the 3-day growth also consisted of a greyish-white, confluent growth which microscopically resembled the

3-day growth produced on AJA medium containing no serum. There was, however, a higher proportion of L elements (about 50 per cent. of the total cells) in the growth obtained on this medium containing non-immune serum. A few large spherical elements were also present in the stained preparations (Dienes' method) made from the 3-day growth but these were not present in preparations made from the corresponding growth obtained on the AJA medium containing no serum. When the 10-day growth was examined microscopically, L elements constituted less than 1 per cent. of the total cellular elements.

On AJA medium containing immune serum, a greyish-white confluent growth was present by the 3rd day consisting largely of pleomorphic bacillary forms viz. coccobacilli, short swollen rods, giant bacilli (?transitional forms) and long and medium-sized rods. There were also significant numbers of long and short filaments. L elements constituted between 40 and 50 per cent. of the total cellular elements. The predominating L forms were medium-sized spherical elements, although there were large numbers of small and tiny types. A few large spherical elements were also present. The 10-day incubated growth obtained on this medium differed in its microscopic composition from the corresponding growth obtained on the other two AJA media in that on AJA medium containing immune serum,

although there was a reduction in the numbers of L elements, they still constituted between 10 and 15 per cent. of the total cellular elements. The bacillary elements present in this 10-day growth consisted mainly of medium sized, extremely stout rods and many long, swollen rods. Many coccobacillary forms were also present and in addition there were appreciable numbers of transitional filamentous forms.

6. Induction of L forms with immune serum and complement.

0.5 ml of an 18-hour PW culture containing 1.5 x 10^9 bacilli/ml of strain 892/71 was mixed with 0.5 ml of inactivated immune serum (titre 1/1280) and 0.5 ml of reconstituted normal guinea-pig complement. This mixture was incubated at 37°C for 30 minutes before it was plated out on AJA medium containing 10 per cent. horse serum. A control plate of the same medium was inoculated with a few drops of the untreated PW culture. Inoculated plates were incubated at 30°C and examined after 48 hours and 8 days of incubation.

On AJA medium inoculated with treated PW culture, a moist, confluent growth was present within 24 hours. Also present were compact colonies growing within the depths of the agar. The confluent growth consisted mostly of swollen long rods and medium-sized short rods. There were large numbers of small and tiny spherical elements. Medium-sized, spherical elements were present in moderate numbers. Spherical elements made up about

20 per cent. of the total cells. A few filamentous and transitional forms were also present. The 8-day growth was more profuse and consisted mainly of medium-sized and tiny rods and large numbers of short filaments. Small numbers of L elements were also present. When the colonies growing within the depths of the agar were stained by Dienes' technique they were shown to consist of swollen, barred, medium-sized rods (?transitional forms) and short, barred, serpentine forms (these are transitional elements). These two forms constituted about 60 per cent. of the total cellular elements while the remainder were composed of L elements (mostly tiny, small and medium-sized spherical forms).

A twenty-four-hour culture on the control medium showed a thin film of confluent growth and a few revertant type colonies similar to those shown in plate 29. These revertant type colonies consisted mostly of bacillary forms, but there were significant numbers of L elements, mostly small and tiny spherical elements. These L elements were slightly fewer in number than in the 48 hour confluent growth produced from the treated PW culture. The 8-day growth on the control medium consisted of many transitional forms (serpentine elements and sausage-shaped forms), tiny and medium-sized rods and coccobacilli. A few short filaments and occasional L elements were also present.

7. Effect of haemolysed serum (inactivated and non-inactivated) on L form production.

Eighteen-hour PW cultures of strain 892/71 containing 1.5 x 10^9 bacilli/ml were inoculated into the following media:

(a) AJA medium containing no serum,

(b) AJA medium containing 10 per cent. unhaemolysed inactivated horse serum,

(c) AJA medium containing 10 per cent. haemolysed inactivated horse serum,

(d) AJA medium containing 10 per cent. unhaemolysed horse serum (not inactivated),

(e) AJA medium containing 10 per cent. haemolysed horse serum (not inactivated).

Inoculated plates were incubated at 30°C and were examined after 24 hours and 10 days of incubation.

On AJA medium containing no serum at all, a slightly opaque, confluent growth appeared within 24 hours consisting mostly of long and medium-sized rods and a few small and tiny spherical elements. When this growth was further examined on the 10th day of incubation only bacillary elements and a few short filaments were seen.

On the medium containing inactivated unhaemolysed serum, the growth findings were identical to those found on the control AJA medium described in the preceding experiments (i.e. induction of L forms with immune serum and complement).

On the medium containing inactivated haemolysed serum, numerous revertant type colonies and a small amount of confluent bacillary type of growth was present after 24 hours' incubation. When the revertant type colonies were examined microscopically they were found to consist of large numbers of long and medium-sized rods, small and tiny spherical elements and short filamentous forms. The filamentous forms constituted between 20 and 30 per cent. of the total cellular elements. A few large and medium-sized spherical elements were also present. When the growth was re-examined on the 10th day of incubation, many of the revertant type colonies had coalesced. This growth now consisted mostly of bacillary elements. L forms and filamentous forms were considerably reduced in numbers.

On the medium with unhaemolysed serum which had not been inactivated the growth findings were, in general, similar to those found on the preceding medium except that in the 24-hour growth there were considerably fewer L elements.

Both the macroscopic and microscopic findings of the growth on the medium containing haemolysed serum which had not been inactivated were similar to those found on AJA medium (c), except that in the 10-day growth there were considerably more L elements (small and tiny spherical elements) than on medium (c).

STRAIN 595/72

1. Induction of L forms with glycine.

Twenty-hour concentrated PW cultures of strain 595/72 were inoculated on the following media:

(a) AJA medium containing 10 per cent. horse serum,

(b) AJA medium containing 10 per cent. horse serum and 1.5 per cent. glycine,

(c) AJA medium containing 10 per cent. horse serum and 3 per cent. glycine.

On AJA medium containing only 10 per cent. serum a creamy-white confluent growth had appeared by the 2nd day and this, when stained by Gram's method, was seen to consist mostly of bacillary elements (medium-sized and short rods and coccobacillary forms). A few filamentous forms and well stained, medium-sized, spherical elements were also seen. In Dienes' stained preparation of this 2-day growth there were large numbers of L elements (ranging from tiny spherical elements to large round, pale-staining bodies). The L elements constituted about 30 per cent. of the total cellular elements; apart from a few filamentous forms the rest were bacilli, chiefly short rods and coccobacilli. When the growth was examined microscopically after 4 days' incubation, Gram's smears showed a large number of filamentous forms (between 10-20 per cent. of the total cellular elements). The length of filaments ranged from 6 - 120 μm. There were large numbers of

coccobacillary forms and smaller numbers of long, medium-sized and short rods. A small number of medium-sized spherical elements, coccal forms and moderate amounts of pink staining nondescript material were also present. Dienes' stained preparations of the growth showed the same elements except that there were larger numbers of L elements (mainly the tiny and small spherical elements; large and medium-sized spherical elements were in smaller numbers).

On AJA medium containing 10 per cent. serum and 1.5 per cent. glycine there appeared a greyish-white, confluent growth by the 2nd day of incubation. Gram's smears of this growth showed a preponderance of coccobacillary forms and large numbers of well stained coccal forms. There were also moderare numbers of well stained, medium-sized spherical elements as well as pink staining nondescript material. A number of long, stout rods occurred in chain formation (5-6 rods forming a chain). This growth, stained by Dienes' method, showed large numbers of L elements (large round, pale-staining bodies and large, medium and small and tiny spherical elements, in that order of abundance). The L elements constituted about 30 per cent. of the total cellular elements. Medium-sized rods and coccobacillary forms were the chief bacillary elements. A few filamentous forms, long rods and small amounts of coarse dark blue staining granules were also present by

by the 4th day of incubation. Most of the bacillary elements had transformed as was evident in both Gram's and Dienes' stained colony preparations. In Gram's smears small numbers of medium-sized and short rods, coccobacillary forms, short filaments, coccal forms and small spherical elements were present. There were large amounts of pink staining nondescript material. Dienes' stained preparations of the same growth showed L elements comprising between 70 and 80 per cent. of the total cellular elements. The main L elements present were small and tiny spherical bodies with considerably fewer large and medium spherical elements. A few large round, pale-staining bodies and filamentous forms were also present. The bacillary elements (short rods and coccobacilli) constituted the rest of the cellular elements. On AJA medium containing 10 per cent. serum and 3 per cent. glycine there was hardly any visible growth after 2 days' incubation and therefore no microscopical examination was carried out. The 4-day incubated growth on this medium appeared as a translucent, patchy, granular L type growth. Gram's smears of this showed only a few medium-sized and large spherical elements (moderately distorted). Most of the smear showed only pink staining nondescript material. Dienes' stained preparations showed the growth to consist almost entirely of L elements. The large round, pale-staining bodies and large spherical

elements were the most numerous but there were moderate numbers of the medium-sized elements and only a few small spherical elements. Tiny spherical elements, filamentous forms and rods were absent.

2. Induction of L forms with immune serum.

Twenty-four-hour concentrated PW cultures of strain 595/72 were inoculated into the following media:

(a) AJA medium containing no serum,

(b) AJA medium containing 10 per cent. non-immune rabbit serum,

(c) AJA medium containing 10 per cent. immune rabbit serum having a titre of 1/2560.

Inoculated plates were incubated at 30°C and were examined on the 3rd and 10th days of incubation.

A thick, greyish-white, confluent growth appeared by the 3rd day of incubation on AJA medium containing no serum. Gram's smears of this growth showed large numbers of long, medium-sized and short rods. Many transitional forms, coccal and ovoid bodies and large amounts of pink staining nondescript material were also present. However, in Dienes' stained preparations of this growth (see plate 44), approximately 40 per cent. of the total cellular elements consisted of altered forms (i.e. L elements and transitional forms). The L elements consisted of spherical bodies, their sizes ranging from the tiny forms to the large round, pale-

staining bodies. A number of the larger L elements i.e. the large spherical elements and large round, pale-staining bodies were slightly distorted. The transitional forms consisted mostly of grossly swollen filamentous forms, much fewer in number than the L elements. Many of the L elements particularly the smaller varieties were more intensely stained than the rest of the cellular elements. No appreciable changes in the gross appearance of the growth were noticed when the plates were examined on the 10th day. Gram's smears of this 10-day growth showed it was made up mainly of large numbers of grossly swollen, long, medium-sized and short rods. A number of coccobacillary forms, small and large spherical bodies (some staining more intensely than others) and some pink staining nondescript material was also present. In Dienes' stained preparations of the 10-day growth the same elements found in the Gram's smear were also present. L elements (mostly small and medium sized) consisted of only 10 per cent. of the total cellular forms.

On AJA medium containing both immune and non-immune serum a confluent, greyish-white growth was present by the 3rd day of incubation. The microscopic composition of this growth on both these media was not strikingly different from that on the AJA medium containing no serum, except that there were proportionately less transitional forms and a slight increase in the number of medium-sized

spherical elements. Likewise the microscopical composition of the 10-day growths obtained on AJA media containing immune and non-immune sera were almost identical, L elements constituting only about 10 per cent. of the total cells present. A feature that was particularly noticeable was that more of the spherical elements withstood Gram's staining in the 10-day growth than in the 3-day growth, denoting the acquisition of some degree of osmotic and mechanical resistance.

STRAIN 1236/72

1. Induction of L forms with glycine.

Twenty-four-hour concentrated PW cultures of strain 1236/72 were plated out on the following media:
(a) AJA medium containing 10 per cent. horse serum,
(b) AJA medium containing 10 per cent. horse serum and 1.5 per cent. glycine,
(c) AJA medium containing 10 per cent. horse serum and 3.0 per cent. glycine.

On AJA medium containing 10 per cent. serum a confluent, opaque, greyish-white growth appeared by the 4th day of incubation. Gram's smears of this revealed pleomorphic bacillary elements, ovoid bodies and a small number of short filaments and small and medium-sized spherical elements. Moderate amounts of pink staining nondescript material were also present. The growth, when stained by Dienes' technique, was

seen to consist of about 30 per cent. L elements (mostly tiny and small spherical elements with a few of the medium-sized forms). When the growth was examined microscopically on the 18th day it consisted mostly of coccobacillary forms with a few medium-sized and long swollen rods. The L elements still constituted about 30 per cent. of the total cellular elements.

On AJA medium containing 10 per cent. serum and 1.5 per cent. glycine a patchy, granular L type growth and a few well isolated, moist colonies were present. The patchy granular L type growth, when stained by Gram's method, showed no cellular forms, but in Dienes' stained preparations large numbers of L elements were present. The large round, pale-staining bodies and the large spherical elements were the main L elements but there were also a few medium-sized and tiny forms. There were, however, a few bacillary forms which were not revealed in Gram's smears. The moist colonies were composed of the same L elements and a small number of bacillary forms. This plate was not examined on the 18th day due to the appearance of contaminants.

On AJA medium containing 10 per cent. serum and 3 per cent. glycine a very translucent, faint, granular growth was present by the 4th day of incubation. Gram's smears of this failed to reveal any cellular forms except pink staining nondescript material, but in Dienes' stained preparations the growth consisted mostly of large round

pale-staining bodies which were very granular along with large and small spherical elements. Tiny spherical elements, filamentous and bacillary forms were completely absent. When the plates were re-examined on the 18th day the growth was as sparse as on the 4th day. Gram's smears showed no identifiable cellular forms and Dienes' stained preparations failed to reveal any stained forms but there were a few unstained large and medium-sized spherical elements. These findings suggested that the growth had hardly developed beyond the 4th day of incubation.

2. Induction of L forms with immune serum.

Twenty-four-hour concentrated PW cultures of strain 1236/72 were plated out on the following media:

(a) AJA medium containing no serum,

(b) AJA medium containing 10 per cent. non-immune rabbit serum,

(c) AJA medium containing 10 per cent. immune rabbit serum with a titre of 1/2560.

Inoculated plates were incubated at 30°C and were examined on the 3rd and 10th days of incubation.

On AJA medium containing no serum a thick, dry, confluent, greyish-white growth had appeared by the 3rd day of incubation. Gram's smears of this showed pleomorphic bacillary forms with a preponderance of short and medium-sized swollen rods. Moderate amounts of pink nondescript material and a few faintly stained

medium-sized spherical elements were also present. This growth stained by Dienes' technique also showed the same bacillary forms as seen in the Gram's smears but between 20 and 25 per cent. of the total cells were made up of L elements. Small and medium-sized spherical elements were themost numerous of the L forms with large and tiny forms in smaller numbers. There were small numbers of blue staining granules. On the 10th day the chief cellular elements were still the bacillary forms (mostly coccobacillary and short swollen rods) but with large numbers of tiny spherical elements and only a few small and medium-sized spherical forms.

On AJA medium containing immune and non-immune serum there appeared by the 3rd day of incubation a moist, confluent, opaque, greyish-white growth which in Gram's smears showed mostly swollen coccobacillary and short swollen rods. Medium-sized rods and coccal bodies were present in moderate numbers. A few of the larger L elements and some pink staining nondescript material were also present. When this growth was stained by Dienes' technique almost 50 per cent. of the cells were L elements. In some areas of the microscopic field the L elements constituted about 70 per cent. of the cells present but in other areas this proportion was as low as 20 per cent. The L elements present ranged from the tiny spherical forms to the large forms. A few unstained large round,

pale-staining bodies could also be identified. Filamentous forms were only encountered occasionally. The 10-day growth showed a definite increase of the bacillary forms but L elements were still present in substantial numbers. More of the L elements in the 10-day growth withstood Gram's staining than those in 3-day growth. The L elements consisted mainly of the tiny spherical elements. The large spherical elements and the large round, pale-staining bodies were absent.

3. Induction of L forms with immune serum and complement.

0.5 ml quantities of an 18-hour PW culture of strain 1236/72 containing 1.5×10^9 bacilli/ml were each mixed with 0.5 ml of three inactivated immune rabbit sera with titres of 1/320, 1/640 and 1/1280. To each of these mixtures was added 1.0 ml of reconstituted normal guinea-pig complement. The mixture of bacilli, immune serum and complement were allowed to react at 37° C for 30 minutes after which they were plated out on AJA medium containing 10 per cent. serum. AJA medium with and without serum (controls) were seeded with untreated bacillary cultures. Inoculated plates were incubated at 30°C and examined after 48 hours and 8 days of incubation.

On control media there developed within 48 hours a moist opaque, confluent growth together with a large number of compact colonies growing into the agar

beneath this confluent layer. Agar blocks bearing both the confluent growth and deep colonies were stained by Dienes' method and showed large numbers of L elements which constituted between 30-40 per cent. of the total cellular elements. There were slightly more L elements in the control medium containing serum. There were large numbers of coarse blue staining granules. Short and medium-sized rods were the main bacillary elements. When the growth was examined microsopically on the 8th day, the confluent growth consisted mostly of bacillary forms (coccobacilli, short and medium-sized rods) and small amounts of pink staining nondescript material. The deep colonies (from the control medium containing serum) when stained by Dienes' method showed mostly medium-sized swollen rods, swollen short filaments and a few transitional rod and filamentous forms. Tiny, small and medium-sized spherical elements still constituted about 10 per cent. of the cells seen.

On AJA medium inoculated with bacilli exposed to immune serum (titre 1/320) and complement a slightly opaque, confluent growth was present by the 48th hour of incubation consisting mostly of medium-sized rods or coccobacillary forms. No large or medium sized spherical elements were present but there were small numbers of tiny spherical elements. The growth was not further examined because of contamination.

On AJA medium inoculated with bacilli exposed to immune serum with titre of 1/640 and complement, the growth that developed after 48 hours of incubation was moderately opaque and confluent. Microscopically it consisted mostly of medium-sized rods and L elements were only occasionally encountered. By the 8th day the growth had become more opaque and consisted mainly of coccobacillary forms with occasional transitional and L elements.

AJA medium inoculated with bacilli exposed to immune serum with a titre of 1/1280 and complement showed also a moist, moderately opaque, confluent growth by the 48th hour of incubation. Microscopically it consisted mostly of medium-sized rods. A few tiny, small and medium-sized spherical elements were also present. When the plates were re-examined on the 8th day, the growth had become more opaque and was more profuse. It now consisted mostly of medium-sized rods, large numbers of coccobacillary forms and transitional rod forms. Although a small amount of pink staining nondescript material was demonstrable in Gram's smears, no L elements were seen in Dienes' stained preparations.

MICROSCOPIC COMPOSITION OF L, HETEROMORPHIC L AND REVERTANT TYPE COLONIES

L colonies

L colonies contained no normal bacillary forms and consisted of a variety of altered forms which were made of spherical elements, large round pale-staining bodies and granular and transitional elements. In addition, distorted forms of the large spherical elements and large round bodies were often present in these cultures (see plates 35 & 40). With Dienes' staining technique most of the altered elements took on a purple or sky-blue colour. The spherical forms constituted the most predominant of the L elements in L colonies and their sizes varied from 5-20μm in diameter. The small spherical elements measured from 1-5μm in diameter. The tiny and small spherical elements occurred mostly in older colonies and in field strains subjected to the action of transformative agents; they also occurred naturally when field strains were plated out on AJA medium containing no inducing agents. The small and tiny spherical elements were not seen to contain granules when examined under the highest magnifications of the light microscope. They were generally more resistant to mechanical distortion than the larger spherical elements. The medium-sized spherical elements measured from 5-10μm in diameter

while the large ones measured from 10-20μm in diameter (see plates 16, 32, 43 & 52). Both these forms occurred in young cultures and with increasing age of the colonies they diminished in numbers, particularly the larger varieties. The large spherical forms were prone to mechanical distortion. Many of the large and medium-sized spherical elements contained granules. The large and medium sized spherical forms differed from the smaller varieties in their staining properties. Some of the large and medium sized forms stained more intensely than others by Dienes' staining technique. This difference in staining was less marked in the smaller elements. In Dienes' stained preparation the tiny spherical forms sometimes stained more intensely at the periphery with a clear central portion. A similar staining characteristic was also seen in some Dienes' stained preparations of the large L elements (see plate 32). It should be mentioned that the apparent vast differences in the intensity of staining of the spherical elements shown in the black and white microphotographs is due in part to the fact that some of the spherical elements stained sky-blue while others were stained purple.

Most of the spherical elements were destroyed by Gram's method and appeared as pink staining nondescript material but in many instances spherical elements could be demonstrated by this staining technique (see plates

25 & 45). The small, medium and large spherical elements appeared as Gram negative spheres. The large spherical elements were often misshapen and were the least easily demonstrated of the spherical elements. The tiny spherical elements in Gram's smears appeared as Gram negative coccal bodies.

The large round pale-staining bodies (see plates 32, 35, 40 & 52) differed from the spherical elements in their size and staining characteristics. These bodies usually measured from 20-25μm but large forms up to 50μm were also encountered. They stained light blue or slate grey by Dienes' staining technique. The large round bodies were never demonstrated by Gram's smears and appeared as pink staining nondescript material. They were the most susceptible of the L elements to mechanical distortion. They were the earliest elements to appear in bacillary cultures exposed to the action of L inducing agents and were also the first to disappear with progressive ageing of the cultures. Their occasional presence in older cultures was due to late transformation of some of the bacillary elements. Most of the large round bodies contained granules and were also capable of producing rods (see plate 40).

Granules in L cultures were of two types, namely a fine variety which took on a faint purplish or light blue colour when stained by Dienes' method and the coarser, larger dark blue granules. The fine lightly

stained granules nearly always occurred intracellularly in the medium-sized and large spherical elements and large round pale-staining bodies. The coarse dark blue granules occurred both intracellularly and extracellularly (see plates 36, 40 & 44). These coarse dark blue staining granules were found intra-eellularly in large spherical bodies and large round pale-staining bodies and in some transitional filamentous and bulbous forms.

Transitional elements in L cultures could be broadly classified into 2 groups. The first group consisted of the transitional rod forms, which were yeast-like bodies, rugby-ball-shaped forms, sausage-shaped bodies, club-shaped long distorted rods, giant swollen bacillary forms, ovoids and short serpentine forms (see plates 12, 44, 45 & 46) and were less commonly encountered in L cultures. The second group were transitional filamentous forms consisting of abnormal filaments and serpentine forms. The degree of abnormality of the filaments varied from slight uniform thickenings to gross enlargements. Often the filaments ended in bulbous swellings. Some of the filamentous forms appeared stippled; many of the transitional filamentous forms had saccular or spherical enlargements along their length. Included in this group of transitional forms were also serpentine forms, tadpole forms and cells resembling fibroblasts. The

transitional filamentous forms can be best seen in plates 10, 11, 16, 17, 44 & 47.

Heteromorphic L colonies

The microscopic composition of heteromorphic L colonies essentially consisted of the elements found in L colonies but differed from it in that there were considerably fewer L elements in heteromorphic L colonies. Heteromorphic L colonies also contained some bacillary elements that were not present in L colonies. These colonies also contained large numbers of transitional filamentous forms but very few transitional rod forms.

Revertant type colonies

Revertant type colonies consisted predominantly of bacillary and transitional rod forms with fewer of the transitional filamentous forms. L elements were also present but these occurred in very small numbers. Extracellular granules were often absent. The various elements present in revertant cultures are shown in plate 13.

INDUCTION OF WALL DEFECTIVE VARIANTS IN LIQUID MEDIUM

Production of spheroplasts

Spheroplasts were produced when 37^{o}C incubated liquid cultures of Salm. gallinarum strain 9S were treated with penicillin. The bacillary forms were

grown in AJB at 37°C for periods varying from 3 hours 40 minutes to 5 hours 25 minutes following which penicillin was added in concentrations ranging from 4,000 to 10,000 units/ml. After the addition of penicillin the treated cultures were reincubated at 37°C and at periodic intervals wet unstained preparations of the culture were examined by phase microscopy. Bacillary forms grown under identical cultural conditions in AJB but without the addition of penicillin served as control.

Broth cultures of Salm. gallinarum strain 9S incubated at 37°C for 3 hours and 40 minutes to 5 hours and 25 minutes showed mostly rods measuring 1-2μm x 0.5μm. A few short filaments measuring 6.0μm x 0.5μm were sometimes present. When penicillin was added to these cultures (4,000 to 10,000 units/ml), most of the bacillary rods became swollen within the first hour after the addition of penicillin, but no spheroplasts were observed. Between the second and third hour after the addition of the penicillin, many of the rods were fragmented and cultures consisted of pleomorphic rods. Spheroplasts were observed in penicillin-treated cultures after 18-24 hours incubation. These cultures appeared slightly turbid and had small numbers of spheroplasts and large numbers of rod forms. The sizes of these spheroplasts ranged from 2-10μm in diameter, but the most commonly occurring types were

those with diameters of 3-5μm. Wet preparations made from treated cultures incubated for 48 hours showed a slightly higher number of spheroplasts than penicillin-treated cultures incubated for 24 hours, but the predominant cells present were still the bacillary rod forms. Penicillin-treated broth cultures examined between the 6th and 10th days of incubation had the highest numbers of spheroplasts. In these 6 to 10 day incubated penicillin-treated broth cultures spheroplasts were the most predominant elements (see plate 48). With further incubation tiny rods began to appear and the number of spheroplasts began to decrease. There were also large numbers of damaged spheroplasts (crenated forms, half spheres, and misshapen spheres) and cell debris. Broth cultures to which no penicillin was added did not produce spheroplasts. Occasionally a spheroplast was seen in non-treated 7-10 day incubated broth cultures.

Spheroplasts were also formed when heteromorphic L or revertant type colonies obtained on AJA medium containing penicillin were transferred to AJB containing 500-10,000 units/ml penicillin.

When heteromorphic L colonies or revertant type colonies were subcultured into AJB containing 1,000 units/ml penicillin there were more spheroplasts than rod forms; this preponderance of spheroplasts over the rod forms were noticeable within 24 hours of adding

penicillin. In AJB containing 500 units/ml penicillin and inoculated with heteromorphic L or revertant type colonies smaller numbers of spheroplasts were present when these broth cultures were examined at 18-24 hours after the addition of penicillin.

Irrespective of the concentration of penicillin, the peak of spheroplast formation appeared to be between the 5th and 10th day after the addition of penicillin. These broth cultures also contained a variety of other cell types namely filaments, filaments with one or more spherical swellings along their length, tadpole-shaped forms, long rods with terminal or central spherical enlargements, and racquet-shaped forms. The spheroplasts produced by inoculating heteromorphic L or revertant type colonies into AJB containing penicillin persisted up to 27 days, whereas those produced by inoculating bacillary colonies into AJB containing penicillin seldom persisted more than 16 days. Heteromorphic and revertant L colonies transferred to AJB containing no penicillin rapidly reproduced bacillary forms.

Bacillary colonies grown in PW failed to produce spheroplasts despite the addition of penicillin in concentrations ranging from 4,000 to 10,000 units/ml penicillin. Slightly turbid 3-4 hour incubated PW cultures of strain 9S became clear within 18-24 hours after the addition of penicillin. Wet smears made from

18-24 hour treated PW cultures showed mostly cell debris and very few intact bacilli, but no spheroplasts or filaments were produced. Even after 2 weeks' incubation the broth did not become turbid and wet preparations never showed any spheroplasts but just a few bacillary rods and cell debris. PW cultures to which no penicillin was added showed mostly rods and occasional short filaments but, after the fifth day of incubation, larger numbers of filaments and small numbers of ovoid cells, yeast-like bodies, dumb-bell-shaped and racquet-shaped forms were present.

Heteromorphic L and revertant colonies also failed to produce spheroplasts when inoculated in PW containing 4,000 to 10,000 units/ml penicillin.

When spheroplasts produced by inoculating heteromorphic L colonies into AJB containing 500 units/ml penicillin were transferred to AJB containing no penicillin and incubated, they transformed into bacillary rods of normal morphology within 24 hours, but when the spheroplasts were transferred to AJB containing 1,000 units/ml penicillin most of them lysed and the broth remained clear up to the 10th day of incubation. However when spheroplasts produced in AJB containing 500 units/ml penicillin were transferred to AJB containing no penicillin, incubated at 37^{o}C for 8-10 hours and penicillin (1,000 units/ml) then added, large spheroplasts were produced, but most

of them disappeared by the 8th day of incubation; wet preparations of the 8-day growth showed mostly bacillary rods and short filaments and a few pear-shaped bodies.

Production of L forms in liquid medium.

L forms were produced in liquid medium by inoculating L colonies into AJB containing penicillin. The process of growing L forms in liquid medium involved the gradual adaptation of L colonies to grow initially on AJA medium in the presence of high concentrations of penicillin (not less than 1,000 units/ml) and then adapting them to grow in AJB containing 10 per cent. serum and 1,000 units/ml penicillin.

Bacillary forms of strain 9S were subcultured on AJA medium containing 100 units/ml penicillin and 10 per cent. horse serum. Heteromorphic L and revertant colonies obtained in this medium were twice serially passaged on similar medium and twice on AJA medium containing 200 units/ml penicillin and 10 per cent. horse serum which resulted in the production of heteromorphic L colonies. These were then subcultured once on AJA medium containing 400 units/ml penicillin and 10 per cent. horse serum. This procedure resulted in getting heteromorphic L colonies low in transitional and bacillary forms. These well developed heteromorphic L colonies were then subcultured on AJA medium containing

1,000 units/ml penicillin and 20 per cent. horse serum. The L colonies thus obtained were further serially passaged on similar medium also containing 1,000 units/ml penicillin and 20 per cent. horse serum, before they were subcultured to AJB containing 10 per cent. horse serum and 1,000 units/ml penicillin. This subculture was effected by transferring agar blocks bearing the L growth to the liquid medium. Good growth of L forms also occurred in the liquid medium by inoculating it with washings of L growth obtained from the solid medium. All inoculated AJB were incubated at 30°C. No growth occurred in the inoculated AJB and it remained clear until the 12th day of incubation but, when the AJB cultures were examined on the 15th day, small amounts of flakes were seen which increased with further incubation. These flakes remained sedimented to the bottom of the vessel and the supernatant AJB was clear up to the 3rd week of incubation. This flaky, sedimented growth in the 15th-18th day of incubated AJB (containing 10 per cent. horse serum and 1,000 units/ml penicillin) stained by Gram's method showed Gram negative branching rhizoid forms, irregular and Y-shaped elements, bulbous forms and pink staining nondescript material (see plate 49). Some of the forms resembled the 'T' elements produced by passaging of L colonies on AJA medium containing 20 per cent. horse serum and 1,000 units/ml penicillin (see pages 249,250 and plate 30). When the

L growth in AJB containing penicillin was incubated at 30°C for periods exceeding 3 weeks the broth turned slightly turbid and Gram's stained preparations of the deposit showed many small spherical bodies, granules and branched bodies with knob-like enlargements and delicate branching filaments with swellings (see plate 50). These structures were present also in wet preparations examined under phase microscopy.

L colonies subcultured into AJB containing 10 per cent. horse serum but no penicillin, produced uniform turbidity and cloudiness of the AJB after overnight incubation but after 3 days of incubation small amounts of flaky growth could be seen. However, when this 3-5 day AJB showing both turbidity and flaky growth was stained by Gram's method it showed short swollen bacillary rods, short filaments, spherical elements, rhizoid and Y-shaped bodies (T elements) and bulbous and saccular forms. These microscopic findings suggested that the growth was made up of both stabilised L elements and bacillary forms.

L colonies subcultured into AJB containing 10 per cent. horse serum and 10,000 units/ml penicillin failed to grow. The AJB remained clear and was devoid of any sedimented growth.

The growth of L forms in AJB was stabilised by serially passaging the flaky growth 4-6 times in AJB containing 10 per cent. horse serum and 1,000 units/ml

penicillin and incubating the inoculated AJB at 30°C; serum was found to be not necessary for the development of L forms in liquid medium nor for their stabilisation in this medium. Although L forms in liquid medium could be produced in AJB incubated at 37°C, this temperature favoured reversion to the bacillary phase particularly with prolonged incubation. An incubation temperature of 30°C was also more favourable for the induction of stabilisation of L forms in liquid medium.

L forms stabilised in penicillin-serum broth were able to propagate as L forms in AJB containing no penicillin or serum, in hypertonic nutrient broth, and in 20 per cent. peptone water. Established L forms obtained in AJB could be made to revert if they were serially passaged between 3-4 times in AJB containing no penicillin. Even after 4 passages in AJB without penicillin L elements and other cell wall defective forms were present in small to moderate numbers. Reversion of L forms in liquid medium was characterised by the medium changing colour and becoming very turbid.

Stabilised L growth in AJB when subcultured to AJA medium (solid medium) containing 10 per cent. horse serum but no penicillin produced well developed L growth. These colonies were composed mostly of small and tiny spherical elements and a few medium-sized spherical bodies. Also present in significant numbers were branching filamentous forms, aster-like bodies,

irregularly shaped and bulbous forms, and rhizoid bodies. Many of these bodies resembled the L elements.

With the 9R strains it was more difficult to obtain pure growth of L forms in liquid medium. L colonies of strain 9R which had been serially passaged 3-4 times on solid AJA medium containing 10 per cent. horse serum and 1,000 units/ml penicillin when subcultured in AJB medium containing 10 per cent. horse serum and 1,000 units/ml penicillin produced both flaky growth, uniform turbidity and cloudiness of the AJB after 7 days of incubation. Gram's smears made from the centrifuged deposits of this growth revealed Gram negative swollen long, medium sized and short rods, short filaments, transitional rod forms and filamentous forms, spherical elements (principally the small and tiny varieties), irregularly shaped forms, Y-shaped and branching rhizoid bodies, bulbous and aster-like elements (T elements).

However, when L colonies of 9R (i.e. L growth which had been serially passaged on AJA medium containing penicillin) were subcultured into AJB containing 10 per cent. horse serum and 10,000 units/ml penicillin, no turbidity occurred but after 7-10 days of incubation a small amount of flaky growth developed. With further incubation the amount of growth did not increase appreciably but when a few drops of the AJB containing the flaky growth were subcultured to AJB containing no

penicillin, turbidity as well as a small amount of flaky growth was produced; decreasing the penicillin concentration from 10,000 to 5,000 units/ml in the AJB substantially increased yields of flaky growth in the liquid medium. Further serial transfers in penicillin containing AJB (5,000 units/ml) stabilised the L growth.

FILTRATION

Experiments to determine the filterability of L phase of Salm. gallinarum were carried out with only the laboratory strains 9S and 9R. The results of the filtration experiments are as follows:-

(a) Well developed heteromorphic L colonies of strain 9S containing L elements (large, medium-sized and a few small spherical bodies), transitional forms and small numbers of bacilli were washed off from a solid AJA medium in either AJB or 40 per cent. sucrose solution. A uniform suspension of the growth was obtained by shaking the culture in the liquid medium. Approximately 10.0 ml of the suspension was filtered through a syringe fitted with an HA 0.45µm Millipore filter membrane. A few drops of the filtrate were allowed to drop on to several plates of AJA medium containing 1,000 units/ml penicillin and 10 per cent. horse serum. The unfiltered growth was inoculated on to several plates of the same medium, and as an additional control,

suspensions of bacillary growth of Salmonella gallinarum strain 9S were also filtered through an HA 0.45µm Millipore filter membrane and the filtrate cultured directly on AJA medium containing the same concentrations of penicillin and serum. All the inoculated plates were incubated aerobically at 30°C in a moist chamber to prevent drying of the medium. When the plates were examined after 7 days of incubation it was observed that the plates inoculated with filtrates of heteromorphic L colonies and plates inoculated with filtrates of the normal 9S bacillary colonies showed no colonial development whereas on the plates inoculated with unfiltered AJB suspensions containing the heteromorphic L colonies, a patchy granular growth was present. This growth showed mostly well developed L elements, moderate numbers of serpentine forms and T elements. Many of the larger spherical elements were coarsely granulated. With further incubation (17 days) the growth on this plate became more profuse and opaque and consisted microscopically of the same elements but now there were proportionately more L and T elements with an almost complete absence of the serpentine forms. There were no normal bacillary forms. In the plates inoculated with filtrates no visible growth was present when they were examined at the 17th day of incubation and they remained sterile after 3 weeks of incubation.

With the 9R strains filtration experiments were

carried out as described for the 9S strains. Well developed 6-day-old confluent heteromorphic L growth from a solid medium was harvested with AJB or 40 per cent. sucrose solution to give a uniform suspension. Approximately 10.0 ml of this suspension was filtered through an HA 0.45μm Millipore filter membrane. The filtrate and unfiltered AJB suspensions were plated out on AJA medium containing 1,000 units/ml penicillin and 10 per cent. horse serum. Inoculated plates were incubated aerobically at 30°C in a moist chamber. After 7 days incubation plates inoculated with filtered growth showed no growth and the plates inoculated with the unfiltered growth showed only a very faint patchy granular growth. When the plates were examined 7 days later no growth was present in the plates inoculated with filtered growth whereas in the plates seeded with the unfiltered growth there were more areas showing patchy granular growth but still the growth was very scanty. After 3 weeks' incubation plates inoculated with filtered growth remained sterile and in the plates inoculated with the unfiltered growth no further colony development had taken place. When the growth from the latter was stained by Gram's technique it showed mostly pink staining nondescript material, a few L elements and some grossly swollen bacillary forms. The colonies did not take up Dienes' stain suggesting that the growth had died out.

In a subsequent experiment an aged reverting L growth consisting mostly of swollen bacillary forms, coccobacilli and a small number of spherical elements (chiefly tiny spherical elements) from a solid medium was filtered through an HA 0.45µm Millipore filter membrane. The filtrate and unfiltered suspensions were plated out on AJA medium containing 1,000 units/ml penicillin. The inoculated plates were incubated aerobically at 30°C in a moist chamber. No growth was present in the plates inoculated with the filtrate when they were examined after 7 days' incubation, but in the plates inoculated with the unfiltered suspensions a faint confluent growth was present. This growth consisted of L elements (predominantly the tiny spherical elements), blue staining coarse granules and a small number of coccobacillary forms. When the plates were examined after 25 days' incubation a thick confluent growth was present in the plates inoculated with the unfiltered suspension. Microscopically this growth was seen to consist of the same elements as were present as in the 7-day incubated growth and in addition there were short rods and filaments. The proportion of bacillary elements to the L forms was considerably greater in this 25-day incubated culture suggesting that reversion was in progress.

On the other hand in the plates inoculated with the filtrate a patchy granular L type growth was present,

consisting entirely of L elements (extremely large round pale staining bodies), spherical elements of all sizes and blue staining coarse granules. There were no bacillary or transitional forms.

(b) The filterability of L elements was tested also by the ability of these forms to grow through the pores of filter membranes.

On several plates of 20 per cent. serum AJA medium containing 400 and 1,000 units/ml penicillin HA 0.45μm Millipore filter membranes were placed. Agar cubes bearing heteromorphic L colonies derived from strain 9R were placed face downwards on these filter membranes and the plates incubated aerobically at 30°C in a moist chamber. After 5 days' incubation 2 of the inoculated plates were removed from the incubator and the filter membrane was gently lifted off the agar to examine for growth. No colony development was observed in these 5 day incubated plates. On the 11th day of incubation another 2 plates were examined and found to have no growth. The remaining plates were examined on the 14th and 16th days of incubation but none of them showed any evidence of growth. Control plates of the same medium were set up by placing agar blocks bearing the heteromorphic L growth directly on to the agar surface without filter memrbanes and after incubation they showed numerous L colonies many of them depicting the classical

morphology.

In another series of experiments agar blocks bearing L colonies (derived from strain 9R) grown on a penicillin-serum AJA medium (10 per cent. serum and 1,000 units/ml penicillin) were placed face downwards on Millipore filter membranes having APD of 0.6μm and 0.8μm. These filter membranes rested on AJA medium containing 10 per cent. serum and 200 units/ml penicillin. The controls in this experiment consisted of AJA medium in which the agar blocks bearing L colonies were placed directly on the medium without an intervening filter membrane. All the inoculated plates were incubated as described in the previous filtration experiments, and were first examined on the 9th day of incubation.

On the control plates a thick opaque bacillary type of growth was present beneath the inoculated agar blocks and areas adjacent to it. Microscopically this growth was seen to consist predominantly of swollen pleomorphic bacillary forms, transitional rod forms, a few serpentine bodies and a small number of tiny spherical elements. There were very few large, medium-sized and small-sized spherical elements. In the plates containing 0.8μm filter membranes, a lightly opaque growth was present on the medium beneath the filter membrane. This growth had almost the same type of cells as found in the growth obtained in the control plates.

In the plates containing the 0.6μm filter membranes a very faint granular type of growth was present beneath the filter membrane.(see plate 51). This growth contained large numbers of spherical elements and although the tiny spherical elements were the most numerous of the L elements, there were also large numbers of larger spherical elements and large round pale-staining bodies. Many of these large round pale-staining bodies, large and medium-sized spherical elements were granular in appearance. Small to moderate numbers of bacillary forms approaching normal morphology were also present. Many of these bacillary forms contained dark blue staining granules (Dienes' staining). There were also large amounts of extracellular dark blue staining granules, and a few serpentine bodies. Bacillary colonies placed on 0.6μm and 0.8μm filter membranes on similar medium failed to produce any growth.

(c) Investigation of the filterability of L forms in liquid medium was carried out mainly with the aim of separating the L elements from the bacillary and transitional forms in the mixed growth obtained with the 9R strain. L colonies that had been passaged 3-4 times on AJA medium containing 10 per cent. horse serum and 1,000 units/ml penicillin were inoculated into AJB containing the same concentrations of penicillin and serum. After 7 days of incubation the growth

contained a mixture of L elements, transitional rods and filamentous forms and bacillary rods. This 7 day growth was filtered through 0.22μm, 0.45μm and 0.8μm Millipore filter membranes. The filtered fluid was inoculated directly into AJB containing no penicillin or serum and also into AJB containing no penicillin but having 10 per cent. horse serum. Unfiltered growth also was inoculated into the same two media. All inoculated AJB were incubated at 30°C. AJB inoculated with filtrates obtained through 0,22μm and 0.45μm filter membranes showed no growth even after 26 days' incubation. AJB containing no serum or penicillin inoculated with filtrates obtained through 0.8μm filter membranes produced turbid growth within 24 hours' incubation. Smears made from this showed it to consist mostly of collections of short and medium-sized rods and large numbers of long filaments. When this growth was examined after 26 days of incubation there were still large numbers of bacilli with fewer filamentous forms. Large amounts of cell debris were also present. In the AJB containing only serum and inoculated with filtrates obtained through 0.8μm filter membranes turbidity was noticed within 24 hours of incubation; smears from this growth showed also pleomorphic bacilli and small to moderate numbers of filaments. However, when smears were made from this AJB after 26 days' incubation, in addition to the bacillary forms there

were small numbers of L elements, (small spherical bodies, tortuous short filamentous forms ending in small round swellings and matted and ramifying delicate filaments with circular swellings). The control AJB containing no serum or penicillin after 26 days' incubation showed only bacillary forms and filaments, whereas in the AJB containing serum, L elements were present but only in small numbers.

BIOCHEMICAL REACTIONS OF SALMONELLA GALLINARUM (BACILLARY, L FORMS AND REVERTANTS)

The following were subjected to biochemical and serological characterisation tests:

(a) All 10 field and laboratory strains of Salm. gallinarum.

(b) L forms derived from strain 9S (9SL) and strain 9R (9RL).

(c) Bacillary forms that had reverted from L forms of strain 9S (9SR) strain 9R (9RR) and strain 592/72 (592/72R). The results of the biochemical characterisation tests are summarised in Table 17 (only the results in which variations were present are tabulated).

There were small differences in the biochemical reactions between the various bacillary isolates of Salm. gallinarum. The bacillary isolates were Gram negative small rods but the field strains were more pleomorphic

than the laboratory strains. All the isolates were catalase positive and oxidase negative. All the bacillary isolates, and revertants did not liquefy gelatin and the indole, malonate, nitrite production, urea, ONPG, phenylalanine, V-P, and the oxidase tests were negative, but the catalase, MR and nitrate reduction tests were positive. The production of H_2S was not detected in strains 37/74, 595/72, 846/71 and 1236/72 and delayed in strains 131/71 and 154/71. There were also differences in the ability of the various strains to ferment carbohydrates. The fermentation of rhamnose was delayed by all strains except by strains 1236/72 and 595/72 which produced acid from this substrate within 24 hours. There were also interstrain differences in the ability to ferment xylose, sorbitol, maltose, trehalose and dextrin. All 10 strains fermented dulcitol but were sucrose and lactose negative. Adonitol, raffinose, inulin starch, salicin and inositol were not acted upon by any of the bacillary isolates, their derived L forms or the revertants.

Strains 9R, 1236/72 and 37/74 agglutinated in 1:500 acriflavine solution indicating that these were the rough variants. All ten strains of Salmonella gallinarum agglutinated strongly in polyvalent Group O Salmonella antiserum (titre 1:80) and in factor 9 Salmonella antiserum (titre 1:320). None of the smooth

strains were agglutinated by the polyvalent H Salmonella antiserum (titre 1:400). The rough strains 9R, 37/74 and 1236/72 however, showed a slight agglutination with Salmonella polyvalent H antiserum but this reaction was considered to be non-specific. All ten isolates were non-motile. No haemolysis occurred on sheep or horse blood agar. On MacConkey agar all strains grew as non-lactose fermenters.

The L forms derived from strains 9S and 9R resembled the parent strains in most of the biochemical properties. The L forms were catalase positive and oxidase negative. While both the parent strains 9S and 9R produced H_2S, the L forms derived from strain 9S did not produce H_2S and the reaction was delayed slightly in L forms of 9R. The decarboxylation of lysine was delayed in the L forms of 9S and 9R. In general the breakdown of carbohydrates by the L forms was slower than in the parent strains, even taking into account the fact that the L forms were incubated at 30°C. Most of the biochemical reactions of the bacillary strains were delayed 24 hours when the cultures were incubated at 30°C.

Rhamnose and galactose were not fermented by L forms derived from strain 9S whereas with xylose a positive reaction was produced by L forms in 7 days while the parent strain produced only a weak reaction.

The revertants arising from L forms differed very

little from the parent bacillary forms. The revertants were very pleomorphic and were serologically identical with the parent strains. The revertants were catalase positive and oxidase negative. The revertants arising from derived 9S L forms gave a weak reaction for H_2S production whereas the parent strains produced H_2S readily. The decarboxylation of lysine was delayed with the revertant forms. Similarly the breakdown of some carbohydrates by the revertants were slower than by the original parent bacillary forms. Revertants arising from L forms of strain 9S produced acid in xylose within 5 days whereas the parent bacillary forms produced only a weak reaction. Likewise with sorbitol, only a weak reaction was noted with the parent bacillary strain whereas the 9S revertants (9SR) produced a clear positive reaction by the 11th day of incubation but in the 9R strain while the parent bacillary strains produced a clear but positive reaction by the 5th day the 9R revertants showed only a weak reaction by the 14th day. All the 3 revertant forms (9SR, 9RR and 592/72R) were dulcitol positive and lactose and sucrose negative.

When biochemical reactions of the L forms were compared with those of the revertants the differences were much less, and these types had more features in common than with their parent strains. Both the L forms and revertants were slow to ferment carbohydrates. In general none of the revertants nor the L forms acquired

any marked significant characteristics not possessed by the parent strains.

(Results summarised in Table 17 on pages 377 and 378.)

TABLE17

Biochemical characters of 10 bacillary, 2L and 3 revertant forms of Salmonella gallinarum

SUBSTRATE	STRAIN NO.														
	9S	9R	37/74	131/71	154/71	595/72	784/71	846/71	892/71	1236/72	9SL	9SR	9RL	9RR	595/72R
CITRATE	-	-	-	-	-	-	-	-	-	-	ND	-	ND	-	-
GLUCONATE	-	-	-	-	-	-	-	-	-	-	ND	-	ND	-	-
H_2S	+	+	-	$+_4$	$+_4$	-	+	-	-	-	-	$\pm$	$+_3$	+	-
KCN	-	-	-	-	-	-	-	-	-	-	ND	-	ND	-	-
ARGININE	$\pm_{21}$	-	-	-	ND	ND	ND	ND	ND	ND	$\pm_{21}$	$\pm_{21}$	-	-	ND
LYSINE	$+_3$	$+_3$	$+_5$	$+_4$	ND	ND	ND	ND	ND	ND	$+_8$	$+_5$	$+_9$	$+_5$	ND
ORNITHINE	-	-	-	-	ND	ND	ND	ND	ND	ND	-	-	-	-	ND
GLUTAMIC ACID	-	-	-	-	ND	ND	ND	ND	ND	ND	-	-	-	-	ND
ARABINOSE	+	+	+	+	+	+	+	+	+	+	$+_4$	+	$+_4$	+	+
RHAMNOSE	$+_{3-5}$	$+_3$	$+_3$	$+_5$	$+_5$	+	$+_2$	$+_3$	$+_3$	+	-	$+_3$	$+_5$	$+_3$	$+_6$
XYLOSE	$\pm$	$+_3$	-	-	-	-	-	-	-	$\pm_{14}$	$+_7$	$+_5$	$+_7$	$+_3$	-
DEXTROSE	+	+	+	+	+	$\pm_{14}$	+	+	$+_4$	+	$+_2$	+	$+_3$	+	+

Table 17 continued

SUBSTRATE	STRAIN NO.														
	9S	9R	37/74	131/71	154/71	595/72	784/71	846/71	892/71	1236/72	9SL	9SR	9RL	9RR	595/72R
FRUCTOSE	+	+	$+_3$	+	+	+	+	$+_2$	$+_2$	+	$+_2$	+	$+_2$	+	+
GALACTOSE	+	+	+	+	+	+	+	$+_2$	+	+	-	+	$+_2$	+	+
MANNOSE	+	+	+	+	+	+	+	+	+	+	$+_2$	+	$+_3$	+	+
GLYCEROL	+	$+_2$	$+_3$	$+_5$	$+_5$	$+_3$	$+_3$	$+_4$	$+_4$	$+_3$	$+_5$	$+_3$	$+_5$	$+_4$	$+_3$
MANNITOL	+	+	+	+	+	+	+	+	+	+	$+_2$	+	$+_2$	+	+
SORBITOL	$\pm_{14}$	$+_5$	$+_4$	$+_5$	$+_5$	-	$\pm_5$	$+_3$	$+_4$	$+_4$	-	$+_{11}$	$+_{10}$	$+_{14}$	-
MALTOSE	+	+	$+_6$	+	$+_5$	$+_{14}$	-	$+_2$	+	$\pm_{11}$	$+_2$	+	$+_2$	+	$\pm_{14}$
TREHALOSE	+	+	$+_{11}$	$+_{11}$	$+_7$	$\pm_{14}$	+	$+_{11}$	$+_{15}$	$+_6$	$+_2$	+	$+_7$	+	-
DEXTRIN	+	+	-	+	+	-	-	$+_6$	$+_7$	$+_{15}$	$+_2$	$+_{14}$	$+_3$	$+_4$	-
DULCITOL	+	+	+	+	+	+	+	+	+	+	$+_5$	$+_2$	$+_4$	+	+

+ = positive reaction $\pm$ = weak reaction ND = not done

$+_2$= fermentation after 2 days 9SL = L forms of 9S

9SR = revertants of 9S 9RL = L forms of 9R 9RR = revertants of 9R

595/72R = revertants of 595/72

STUDIES ON THE ULTRASTRUCTURE OF BACILLARY AND CELL WALL DEFECTIVE VARIANTS OF SALMONELLA GALLINARUM

The ultrastructure of the cell wall of Salm. gallinarum strains 9S and 9R and the derived cell wall defective variants of strain 9S were examined in thin sections with the aim of establishing further morphological evidence of the organisation of their various surface layers.

Bacillary forms.

The electron microscope profile of the bacillary forms of Salmonella gallinarum strain 9S grown in hypertonic broth medium (AJB) are shown in plates 62, 63, 64 & 65. The cell contents of the bacillary forms are surrounded by two separate and superimposed layered integuments as shown in plates 62, 63, 64 & 65. The cell wall is represented as a five-layered structure characterised by three electron-dense lines separated by two electron-transparent areas (see plates 63 & 64). However, under very high magnifications, to the inside of the 3 electron-dense lines there appears another faint electron-dense line which is well demonstrated on some areas of the cell profile (see plate 64). The fourth electron-dense line would appear to represent part of the cell wall proper but in magnifications of x 80,000 and less the cell wall can only be recognised

as a five-layered membrane. The cell wall of Salmonella gallinarum is these electron micrographs shown it to be between 17 to 18 nm thick.

The cell wall is separated from the cytoplasmic membrane by an electron light area (see plates 64 & 65). The gap between the cell wall and the cytoplasmic membrane is very marked and often exaggerated in bacteria grown in a hypertonic solid medium (see plates 66, 67 & 68) while in bacteria grown on isotonic medium the cytoplasmic membrane is more closely applied to the cell wall (see plate 69). The cytoplasmic membrane is represented by the innermost triple-layered integument (see plate 64) having a total thickness of 5.0-7.5 nm.

In hypertonic liquid medium a few of the bacillary forms show a wrinkled cell wall profile (see plate 65) but this is less marked when they are grown on solid hypertonic medium (see plates 66, 67 & 68). However, in bacilli grown on isotonic medium, the wrinkled profile of the cell wall is noticeable (see plate 69).

L forms and cell wall defective variants in liquid medium.

The ultrastructure of L forms and cell wall defective variants grown in liquid medium as shown in plates 70, 71 & 72. The L forms, as shown in plate 70, show no cell wall. They are bounded only by a

cytoplasmic membrane. The cytoplasmic membrane in some of these L forms can be clearly recognised as a triple-layered integument. Occasionally a part of the cell wall layer is retained on some of the L forms and this is shown as an additional electron dense line overlying the cytoplasmic membrane. In such instances part of the circumference of the L cell showed an extra electron-dense line external to the cytoplasmic membrane. A second variety of cell wall defective variants found in L cultures induced in liquid medium were organisms that possessed a modified cell wall (see plates 71 & 72). The modified cell wall, unlike the normal cell wall, showed only 2 electron-dense lines separated by an electron-lighter area. This modified cell wall had a thickness of approximately 7 nm. In some parts of the L form of the second variety the cell wall showed 3 electron dense lines (see plate 72) but the innermost of these was less electron-dense and did not form a continuous layer. The total thickness of the cell wall increased to approximately 16 nm when this extra electron-dense line was present because it included two electron-light areas between the second and third electron-dense lines. The cytoplasmic membrane in these types of L forms was also triple-layered and did not differ in any respect from the cytoplasmic membrane of the first variety of L forms or the bacillary forms. A third variety

of elements present in these cultures were dense rounded bodies occurring free in the medium (see plate 70). The largest of these measured 300nm and the smallest 75nm. These bodies appeared to be filled with ribosomal particles. The smaller bodies were more dense than the larger bodies.

L forms on solid medium.

The L forms induced on glycine-containing medium are shown in plates 73, 74, 75, 76, 77 & 78. Three types of cell wall defective variants are also identifiable in L cultures grown on solid medium. The first type are the L forms which have lost the cell walls completely (see plates 73, 74, 76 & 78) and the second type are cell wall defective variants which retain a modified cell wall (see plate 75). The modified cell wall in these L forms is represented by an electron dense-light-dense structure having a total thickness of approximately 12nm. The cytoplasmic membrane in these appears as two electron-dense lines separated by a single lucent layer and is approximately 7nm thick. In both the cell wall defective variants and the L forms, vesicular structures and dense bodies are present. Many of the vesicles are empty while a number of them contain granular material resembling ribosomal particles. Many of the vesicular structures are surrounded by a 'unit' type membrane (Robertson, 1959). Vesicular and

dense bodies were also present outside the cells (see plates 75 & 76). The smallest of the dense bodies measured 70nm while the largest of them was 450nm. In some L forms the cytoplasm consisted of collections of dense granular ribosomal particles distributed irregularly in the cell (see plate 73) while in others the ribosomal areas are peripherally distibuted with small clusters of them occurring in the central areas (see plates 76 & 78). A third variety of cell wall defective variant found in these L cultures was L forms in which small segments of cell wall were found still attached to the cells (see plate 78), but the major portions of the cell contents were enclosed only by the cytoplasmic membrane. There were also L forms which showed very little internal organisation and appeared practically empty except for a few dense bodies and small clusters of ribosomal particles (see plate 76).

Transitional forms and reverting bacteria.

The electron micrograph profile of a transitional filamentous form is shown in plate 65. The elongated filament shows enlargements at each pole. A layered cell wall measuring 16-17nm thick is present in these forms, but the cell wall shows damage at the poles where the enlargement in the filament is most marked. The cytoplasmic membrane can be recognised in certain

areas as a 'unit' membrane and is approximately 6-7nm thick.

The ultrastructure of a reverting bacterium in an aged L colony is shown in plates 76 & 77. A cell wall showing the characteristic three electron-dense lines is present. The cell wall is wavy in outline and is separated from the cytoplasmic membrane by a wide space. The separated cell wall had a thickness of approximately 15nm. The cytoplasmic membrane shows the typical 'unit' membrane appearance and is approximately 6-7nm thick. In most of the reverting bacillary forms there is a wide gap between the cell wall and the cytoplasmic membrane, and the cell wall appears very undulating (see plates 76 & 77). The nucleoplasmic regions are more organised in these reverting forms (see plate 76). The nuclear region is centrally located and is surrounded by the ribosomal area.

DISCUSSION

The review of the literature on the L forms of bacteria presented in the first part of this work has shown the considerable volume of work which has been reported on their occurrence, induction, propagation and biological properties. While on the one hand the reported findings on the L forms of bacteria are contradictory and controversial, many of the observations in the more recent literature on the nature and character of these organisms are in close agreement and have provided a fuller understanding of bacterial variation. The investigations reported in the second part of this work were intended to define the conditions under which Salmonella gallinarum converted to L forms, their biological properties and their pathogenic potentials.

Except for a report on the production of Salmonella gallinarum spheroplasts by Salton and Shafa (1958) the author is not aware of any recorded investigations on the induction, characterisation and the pathogenic potentials of the L forms of Salm. gallinarum.

In the present work, ten strains of Salm. gallinarum were studied for their ability to transform into L forms, and in two of these the laboratory strains 9S and 9R, a more detailed assessment was carried out to determine the cultural procedures and factors that influenced the production and propagation of L forms under the

influence of penicillin and glycine. The remaining eight strains (referred to as field strains or isolates) were obtained very much later in the course of investigations to examine whether field isolates would respond to the L transformation quite differently from the more established laboratory strains and consequently they were not investigated to the same extent as the laboratory strains.

In the present work it was observed that while it was possible to induce L transformation in all ten strains of Salm. gallinarum with penicillin and/or glycine, L transformation was dependent on a number of factors such as the concentration of the L inducing agents to which bacillary forms were exposed, cultural conditions and the capacity of the different strains to undergo such transformations and methods of induction.

Although L forms could be obtained by treating a broth culture of Salm. gallinarum strain 9S to the action of penicillin and plating the exposed bacilli on a hypertonic medium at 37°C, a high concentration of penicillin was required to bring about such a transformation. With lowered concentration of penicillin in the broth culture only a heteromorphic L growth developed on hypertonic solid medium. It was interesting to note that on an isotonic solid medium such as nutrient agar bacilli exposed to penicillin concentrations of 4,000-40,000 units/ml in broth cultures

produced dwarf bacillary colonies at the higher concentrations and confluent bacillary growth at the lower concentrations, indicating that a part of the population of Salm. gallinarum strain 9S was resistant to the action of penicillin even at such high concentrations to be able to multiply as bacillary colonies on ordinary nutrient agar.. It was also significant to note that the same treated broth cultures were able to give rise ultimately to L colonies on a hypertonic medium suggesting some of the treated cells were converted into osmotically sensitive forms being capable of multiplying only on a hypertonic solid medium. However, when penicillin treated broth cultures were plated on a hypertonic medium containing penicillin further bacillary or L colony formation was dependent not only on the amount of penicillin added to the broth cultures but also on the amount of penicillin present in the propagating hypertonic medium. Complete inhibition of all colony formation occurred when the broth cultures containing very high concentrations of penicillin were subcultured on hypertonic medium containing high concentrations of penicillin, whereas L colonies developed when the initial exposute dose was lowered and only sufficient amount of penicillin was present in the propagating solid hypertonic medium to allow further development of the treated osmotically labile cells. Lowering

the amount of penicillin in the propagating medium favoured the development of heteromorphic L colonies and with further lowering of penicillin concentration in the propagating medium the bacillary type of growth developed. These above findings suggest that L forms develop only at certain optimum concentrations of penicillin which are conducive to their survival in an hypertonic environment. These observations are in agreement with the findings of Kubota et al. (1966) who demonstrated that the production of protoplasts of Strep. faecalis on an hypertonic solid medium was dependent on the concentration of penicillin present in the inducing medium. In their experiments increasing yields of protoplasts were obtained with increasing concentrations of penicillin giving an optimal yield at penicillin concentrations of 6,250 to 12,500 units/ml but further increases in the penicillin concentration depressed protoplast formation and at 100,000 units/ml penicillin concentration no protoplasts could be obtained.

This method of inducing L forms by exposing bacteria to the action of transforming agents in liquid broth cultures and subsequently plating the treated bacilli on appropriate solid media has been reported by Abbate et al. (1973) but they observed that using two strains E. coli, one of Shigella flexneri and two of Klebsiella - Enterobacter no L colonies were obtained by this method,

and that persistence of bacterial growth occurred with the Klebsiella-Enterobacter strains. On the other hand, Godzeski, Brier and Pavey (1962a) induced L growth from a variety of bacteria by exposing broth cultures of the bacillary forms to relatively low concentrations of antibiotics and plating the treated bacilli on solid hypertonic medium. The findings reported here with Salm. gallinarum are in agreement with the observations of Godzeski et al. (1962a) and Abbate et al. (1973) in that while it is possible to obtain L growth by this method, very often untransformed elements (bacillary persistors) or partially transformed (transitional) elements were present in the L growth despite the very high concentrations of penicillin employed. Probably the short period to which the bacilli were exposed to the action of penicillin in the liquid medium were insufficient to convert all the bacilli to osmotically sensitive forms thus enabling them to grow only as L elements on a solid hypertonic medium. This could very well be so as subsequent experiments have shown that, within limits, bacilli exposed to the action of penicillin in a liquid medium produce quantitatively more L elements when plated out on an appropriate hypertonic medium than those exposed for shorter periods. It might be argued that the failure to transform in a liquid medium containing high concentrations of penicillin may be ascribed to resistance

of the organism to penicillin, but it is difficult to arrive at this conclusion because bacilli that had been exposed to such high concentrations of penicillin failed to grow when plated out on a hypertonic medium containing only moderate amounts of penicillin (i.e. less than the amount of penicillin to which they had already been exposed) thus denoting their extreme sensitivity to subsequent exposures to penicillin. On the other hand it cannot be said that none of the bacillary forms exposed initially to the action of penicillin in liquid medium developed resistance because when such bacilli were plated on hypertonic media containing very small amounts of penicillin only heteromorphic and bacillary colonies developed indicating that some of the bacilli had developed partial resistance during their initial exposure to penicillin.

It had been reported by Kandler and Kandler (1956) and Medill-Brown and Hutchinson (1957) that the physiological state of the organism at the time of exposure to the inducing agent affects L transformation. However, in this study with Salm. gallinarum, the addition of penicillin to broth cultures at different growth phases did not markedly affect L transformation, although the number of bacillary forms was slightly reduced in the treated pre-exponential cultures plated on hypertonic medium.

Using the more conventional method advocated by

most workers, untreated broth cultures of Salm. gallinarum were plated out on a hypertonic medium containing varying concentrations of penicillin. While most of the bacillary forms of Salm. gallinarum could be transformed into L elements, particularly on media containing 200-400 units/ml penicillin, the L growth produced was poor and inconsistent while at lower concentrations only a small proportion of the cells transformed into L forms. Equally disappointing results were obtained by diffusing penicillin from wells or troughs in the medium since as when 40,000 units of penicillin were diffused only heteromorphic L growth was obtained. It thus became obvious that penicillin concentrations and hypertonic solid medium were not sufficient to obtain complete L transformation in these laboratory strains of Salm. gallinarum.

Since the stimulatory effect of divalent cations on L transformation and protoplast and spheroplast production has been reported by a number of workers (Weibull, 1956; Lederberg and St.Clair, 1958; Madoff and Dienes, 1958; Shchegolev and Prozorvskii, 1963; Diena et al., 1964; Hamburger and Carleton, 1966a; Davies et al., 1968; Muschel, 1968; Blazevic and Nimmo, 1969; Makemson and Darwish, 1972; Cheng, 1973), media were devised which contained appropriate concentrations of calcium chloride (MC medium) and magnesium sulphate (M medium). No marked stimulatory

effects were obtained in L transformation by the addition of calcium chloride. M media containing both calcium chloride and magnesium sulphate had a stimulatory effect in promoting conversion of bacillary forms to L forms in that penicillin-treated broth cultures of Salm. gallinarum produced proportionately more L elements than the corresponding growth obtained on AJA medium which contained magnesium sulphate but no calcium chloride with the yield of heteromorphic L growth was much reduced on the M media.

The temperature of incubation proved to be an important factor for the transformation of the bacillary phases of Salm. gallinarum into their cell wall defective forms and for their subsequent development. Bacilli exposed to the transformative action of penicillin and incubated at 30°C favoured quantitative transformation into their L phases whereas at 37°C L transformation was incomplete, retarded and inconsistant. Landman and Ginoza (1961) also noted that in Salm. paratyphi, although L growth could be obtained in the temperature range 31-40°C, at 37°C L growth was not consistently produced. The incomplete transformation and retarded appearance of the altered L variant growth of Salm. gallinarum incubated at 37°C could possibly have been the result of a greater loss of penicillin activity during this period of incubation than at 30°C. It was also observed in the present studies that in Salm. gallinarum

the altered variants produced at 37°C reverted more rapidly than did those produced at 30°C. Altenbern (1961 a & b) also made similar observations that not only reversion of L growth to the bacillary state was fostered more readily at 37°C than at 30°C, but also that transformation of the more stable type 3A L colonies to the unstable, easily reverting type 3B colonies in Proteus mirabilis was enhanced. In Salm. gallinarum the more pronounced reversion changes noticed at incubation temperatures of 37°C could probably be explained by the fact that since, at this temperature, transformation to the L phase was incomplete and that, since the altered growth produced at this temperature consisted of a larger proportion of transitional and bacillary elements, reversion was expected to be more pronounced and more rapid than in the corresponding transformed growth containing no bacillary elements and very few transitional forms.

Opinions differ as to the role of mammalian serum for the induction and maintenance of cell wall defective variants from the various species of bacteria. From the short review on this subject presented in the first part of this report it will be noticed that the vast majority of workers in this field have advocated enrichment of the induction medium with serum. For the production of type 3A colonies in the Salmonella spp. Dienes (1949a), Weinberger et al. (1950) and Altenbern

(1961a) have found the inclusion of serum in the induction medium to be absolutely necessary.

In the present work with Salm. gallinarum at no time was it found that serum was absolutely necessary for L transformation nor did the inclusion of serum induce or favour the development of type 3A colonies. However, the inclusion of serum in the induction medium for the transformation of Salm. gallinarum into its cell wall defective forms produced significant results. Induction medium containing 100 units/ml penicillin and 10 per cent. inactivated horse serum inoculated with bacillary forms of the laboratory strains 9S and 9R and incubated at 30°C produced a high yield of patchy granular L type growth within 24 hours of incubation, whereas the same medium but without serum produced only a scanty L growth. A similar but more pronounced effect was noticed when the cultures were incubated at 37°C in that while a faint patchy granular L type growth was produced within 48 hours of incubation on the medium containing serum no growth developed up to the 7th day of incubation on the induction medium devoid of serum. When the penicillin concentration in the induction medium was increased, serum was found to be essential for the development of L growth; thus in the 150 units/ml induction medium, the bacillary forms (incubated at 30°C) failed to produce any growth in the absence of serum up

to the 16th day of incubation, whereas under identical conditions on medium containing 10 per cent. inactivated serum, patchy granular growth developed by the 2nd day of incubation and by the 12th day isolated L colonies depicting the classical morphology were present. With further increase of penicillin (400 units/ml) in the induction media, there was complete cessation of all growth when serum was omitted while L colony development proceeded on media containing serum. From these results it is difficult to conclude what precise role serum plays in the development of L colonies. As it has been shown in the present studies that L colony formation is inhibited when the medium contained high amounts of penicillin and since it is a well established fact that penicillin activity is considerably reduced in the presence of animal proteins, it would appear reasonable to assume that serum in the induction medium reduced the activity of penicillin to the extent of allowing transformed bacilli to develop as L forms which otherwise would have been inhibited by the action of penicillin had not serum been present. Such a conclusion would appear to be valid particularly in view of the fact the L growth was maintained in induction media containing serum with increasing concentrations of penicillin, while in induction medium devoid of serum there was progressive inhibition of L growth.

Increasing the concentration of serum in the

induction medium from 10 to 20 per cent. favoured both qualitative and quantitative transformation changes. These additional beneficial effects on L growth brought about by increases in the serum concentration cannot be attributed solely to the binding of the excess penicillin by serum for if this was so far less free penicillin would have been present in such a medium to bring about L transformation and as such bacillary colonies would have made their appearance. Thus serum would appear to have a nutritive or possibly a stabilising effect on L growth in addition to its detoxifying action on penicillin; alternatively it may be that serum alone was able to convert a portion of the bacterial population into the L-phase conversion being more complete when antibiotic was present (Godzeski, Brier & Pavey, 1962b).

An unexpected effect of serum in certain induction media (M and MAJA medium) was the inhibition of the stimulatory effects of magnesium and calcium ions on L transformation. An observation of this nature has not hitherto been reported and the reason why serum acts so antagonistically in the presence of both these divalent cations must for the present remain speculative. The failure in the present work to produce type 3A colonies in serum medium does not accord with the findings of almost all other investigators studying L transformation in Salmonella on a serum medium. However

Mattman et al. (1969) showed that the morphology of L colonies of Salm. pullorum differed from those of other species of salmonellae.

The effect of inoculum size in relation to L colony formation has not been investigated extensively. Brem and Eveland (1968a) observed that a heavy bacillary inoculum was necessary for the induction of L forms in Listeria monocytogenes. Abbate et al. (1973) noted that the percentage of L forms induced was not only related to the concentration of the inducing agent but also to the size of the vegetative inoculum. These reports prompted the author to determine whether such a relationship existed in the transformation of Salm. gallinarum into its L phases.

In the experiments conducted with Salm. gallinarum strain 9S a bacillary inoculum containing less than 5.5×10^5 cells failed to produce L growth on a penicillin-serum induction medium. With the 9R strains, using similar inoculum sizes and under identical cultural conditions, it was noted that a bacillary inoculum of less than 5.5×10^6 cells failed to produce L colonies. In subsequent experiments broth cultures of strains 9S and 9R plated on inducing media containing a combination of glycine-penicillin and in induction media with glycine alone showed a similar relationship between inoculum size and the ability to form L colonies. Thus these experiments not only

confirmed the findings of previous workers (Brem & Eveland,1968a and Abbate et al., 1973) but also showed the ability of strains within a species to respond differently to L transformation. In spite of the slight differences between these two strains of Salm. gallinarum, it would appear from the present studies that the yield of L colonies from a given inoculum of Salm. gallinarum must be of a very low order (approximately 0.000005 per cent.). Such a low yield of L colonies is in sharp contrast with the findings of Landman and Ginoza (1961) who showed that between 5 and 50 per cent. of Salm. paratyphi could be transformed into the L phase. In Clostridium tetani a yield of 0.0005 per cent. has been reported (Scheibel and Assandri, 1959) and in Pseudomonas aeruginosa between 0.0005 and 3.0 per cent. of the cells were found capable of L transformation (Bertolani et al. 1975). In both these organisms induction of L forms is known to be difficult and this could partly explain why L transformation in Salm. gallinarum strains 9S and 9R was not easy.

The hydrogen ion concentration of the induction medium had a marked qualitative effect on the L transformation of Salm. gallinarum organisms. In the three pH values studied the induction medium with pH 7.2 was found to give the highest degree of L transformation. Yields of L forms were significantly greater in the

medium with pH 7.2 than in the acidic or more alkaline media. Contrary to the observations of Minck and Lavillaureix (1956) and Klieneberger (1936) (an induction medium with a markedly alkaline reaction (pH 8.1)) provided the least favourable environment for L transformation in both of the laboratory strains 9S and 9R. The agar used in most of the induction and propagation media during the course of the present work was of a too highly purified grade to show the presence of any inhibitory substances. Varying the agarose concentration between 0.3 and 0.6 per cent. had no adverse effect on L colony development.

When glucose was autoclaved along with the other media components of the AJA medium it was found to inhibit the development of L growth of Salm. gallinarum considerably. Altenbern (1961a) observed that alkaline oxidation of glucose in the presence of phosphate salts produced substances which were inhibitory for type 3B colonies of Proteus mirabilis. Although the AJA medium used here did not contain added phosphate salts, it is possible that in the organic components used in the AJA medium appreciable amounts of phosphates were present; as the AJA medium had a pH of 7.4, the autoclaving of glucose in the presence of phosphate salts could have liberated toxic substances inhibitory for the development of L forms.

A number of media were investigated for the ability

to support L growth. In all these media penicillin was used as the main L inducing agent.

Although the medium recommended by Makemson and Darwish (1972) was found useful in obtaining L transformation, it never yielded heavy growths of L colonies. Furthermore, the addition of serum to this medium not only suppressed L growth but also caused a marked turbidity of the medium making it hardly possible to study the gross morphology of the L colonies.

L transformation on MAJA medium was unsatisfactory but it did foster heavy bacillary growth of Salm. gallinarum. The MAJA medium lacked yeast extract and glucose, both of which are allegedly important for L transformation and viability. Madoff (1970) and Mattman et al. (1961) have found that yeast extract in the medium enhanced production and propagation of L forms. Similarly, glucose was found to be necessary for the production of Salm. typhi spheroplasts (Diena et al., 1964).

A medium very similar to that used by Kawakami et al. (1970) to isolate unstable L forms of Salm. enteritidis and Salm. typhimurium failed to induce L growth in Salm. gallinarum. Even in the absence of penicillin, this medium supported only a poor growth of the bacillary forms of the organisms. A further disadvantage was the presence of salt precipitates in the agar which interfered with the microscopic examination

of the colonial morphology by Dienes' staining method. This medium contained all the media components used by Kawakami et al. except that the agar, yeast extract and peptones were from different commercial sources.

Attempts to induce L growth of Salm.gallinarum in synthetic and semisynthetic media with penicillin as an inducing agent were unsuccessful. The synthetic medium used was a modification of Medill-O'Kane's medium (1954). Salton and Shafa (1958) also found that the original Medill-O'Kane's medium was unsuitable for the induction of spheroplasts from Salm. gallinarum but, when they increased the salt and Casamino-acids content of the medium, spheroplast production was achieved. In the synthetic medium used in the present work, the salt content was increased as recommended by Salton and Shafa (1958) but the Casamino acid content was reduced and this would probably account for the present failure. The addition of serum to the synthetic medium failed to produce any noticeable improvement.

The AJA medium, which is a modification of Alexander-Jackson's original formula (1954), was found to be the most useful medium for the induction and propagation of cell wall defective variants of Salm. gallinarum. Towards the latter part of the present studies it was found that increasing the sucrose content of this medium AJA(E) was stimulatory

for L transformation particularly in the case of some of the field isolates.

The use of glycine to induce L transformation in Salm. gallinarum gave varying results. A glycine concentration of 1.5 per cent. in the induction medium was insufficient to produce any significant degree of L conversion in the laboratory strains 9S and 9R but, with the addition of serum, a larger proportion of the cells transformed into their L phases. Increasing the glycine content of the medium to 3 per cent. without the addition of serum brought about more complete L transformation but the yields were extremely low. However, when serum was added to the medium containing 3 per cent. glycine, yields of the L growth were considerably improved. The failure to obtain sufficiently satisfactory L conversion in the laboratory strains of Salm. gallinarum is at variance to the findings of Diena et al. (1964) who were able to produce spheroplasts (L forms) in Salm. typhi employing glycine concentrations in their induction medium from 1.5 to 2.0 per cent. Likewise, Want and May (1975) were able to induce L forms in Haemophilus influenzae with as little as 0.7 per cent. glycine in their medium. Diena et al. (1964) have also stated that 3 per cent. glycine was bactericidal for Salm. typhi but in Salmonella gallinarum this does not appear to be so, although L colony production was considerably depressed particularly

when serum was not incorporated into the medium. These findings on Salm. gallinarum confirm the recorded observations of Dienes and Zamecnik (1952) and Jeynes (1957) that it is possible to obtain L transformation with 3 per cent. glycine.

Foremost among other observations in the induction of L forms in Salm. gallinarum was the effect that serum had in fostering the production of glycine induced L forms. Although the usefulness of serum in the production of penicillin induced cell wall defective variants has been discussed earlier it would seem appropriate here to highlight the important role serum played in ensuring survival of the cell wall defective forms. It is possible that if Diena et al.(1964) had incorporated serum in their media they might have been successful in inducing L phases of Salm. typhi with 3 per cent. glycine. Although Dienes and Zamecnik (1952) were successful in obtaining L forms in Salm typhimurium using 3 per cent. glycine in a medium not containing serum they had used 20 per cent. ascitic fluid which might have acted in a similar manner to serum.

In a medium containing serum the combined L inducing effects of glycine and penicillin at low levels (i.e. 1.5 per cent. glycine and 50 units/ml penicillin) were greater than when either of the agents were used singly at their respective concentrations. When the concentration of glycine was increased to 3 per cent.

and that of penicillin was maintained at 50 units/ml, the L growth produced was only marginally superior to that produced by glycine at 3 per cent. concentration; however the growth produced by 50 units/ml penicillin was unmistakably inferior to that produced in the combined penicillin-glycine medium. These findings lend support to the observations of Madoff et al. (1967), Madoff (1970) and Want and May (1975) on the synergistic effect of glycine-penicillin combination in bringing about L transformation in some bacterial species.

Information on the in vitro induction of L forms with inactivated immune serum in sparse. In the studies conducted with Salm. gallinarum strains 9S and 9R using inactivated immune serum with a high antibody content (titre 1:2560), L conversion did not occur but it did induce a considerable amount of pleomorphism in an otherwise normal non-pleomorphic culture. The presence of abnormal numbers of pleomorphic bacilli and transitional filamentous forms suggests that the immune serum interferes to some extent with the normal assembly of cell wall constituents but is not sufficiently potent by itself to bring about more drastic structural cell wall damage. The 9R strain did not appear to be affected by the presence of immune serum in a similar way.

Using a more complete and specific bacteria-antibody-complement system in an in vitro environment,

L transformation in the 9S strain could be achieved in a small proportion of the cells. In the presence of complement immune serum with a titre of 1:1280 was marginally superior in causing L conversion compared with sera with lower titres. It is thus concluded that L transformation is much more readily achieved in the presence of complement when the antibody content of the serum is high. The above observation differs from that of Dienes et al. (1950a) only in as far as L colony production is concerned. Dienes et al. were able to produce L colonies with Salm. typhi whereas with Salm. gallinarum (even using an immune serum with a high titre of 1:1280 in the presence of complement) only heteromorphic L colonies were produced. On the other hand, Brem et al. (1968a) adopting a similar L inducing system in Listeria monocytogenes produced a growth (purported to be L colonies) consisting of elements of the same type present in the heteromorphic L colonies of Salm. gallinarum, produced by complement and antiserum. Brem and Eveland (1968a) also noted that immune serum without complement failed to produce L forms, which is in agreement with the present findings with the laboratory strains of Salm. gallinarum.

The primary role of sucrose in an induction medium is to provide an effective osmotic milieu for the integrity and development of the cell wall defective organisms. Weibull (1953) was among the first to

recognise this requirement which has been confirmed by numerous other workers.

In the studies with Salm. gallinarum strains 9S and 9R, sucrose was used to provide the necessary osmolarity. The concentration of sucrose used for the major part of the present studies was approximately 0.4M and at this concentration it was found to be satisfactory. When the coneentration of sucrose was doubled some of the bacilli were converted into L elements, whereas on the corresponding media containing the usual strength of sucrose no such transformative changes were noticed. The only other known recorded observation is that of Mahony et al. (1971) who found that sucrose was capable of inducing L transformation in Clostridium perfringens.

The ability of haemolysed horse serum to favour L transformation is based on a chance observation on one field isolate of Salm. gallinarum (strain 892/71). Haemolysed inactivated serum was found to stimulate L conversion to a greater degree than unhaemolysed inactivated serum. The observations recorded here were further complicated by the fact that this strain had a natural tendency to produce L forms even on the control AJA medium not containing serum. Because of this the proportion of L forms and other cell wall defective variants to the bacillary elements in the growth produced on medium containing haemolysed and unhaemolysed serum

was used in drawing conclusions in these experiments. Haemolysed serum when used in the non inactivated state produced less L elements but this effect was only transient. There has been no published report that haemolysed serum (non-inactivated and inactivated) can affect L transformation in bacteria.

Differences between strains within bacterial species in the inability to undergo L transformation have been reported by many workers. In the ten strains of Salm. gallinarum used in the present work differences were noted in their capacity to undergo such transformative changes. The differences were both quantitative and qualitative and were first observed when the 9S and 9R strains were investigated for their ability to transform at different incubation temperatures (see experiments described on pages 206 - 233).

On AJA medium containing 100 units/ml penicillin and incubated at 37°C the 9S failed to show any growth but the 9R strain under similar conditions produced a faint patchy granular L growth which remained poorly developed. When serumwas added, both the 9S and 9R strains responded to L transformation more effectively, but the L growth and heteromorphic L colonies formed by strain 9S were better developed and more profuse than those of 9R showing that in the absence of serum the strain 9S was inferior to strain 9R in undergoing L transformation at an incubation temperature of 37°C.

On the other hand under more suitable cultural conditions strain 9S had a greater capacity to transform than 9R. This is further illustrated when the effects of inoculum size on L colony production were studied (see experiments described in pages 223-224). In this experiment it was noted that with strain 9S, L growth could be obtained with bacterial inoculum sizes of 5.5×10^6 and 5.5×10^5 whereas with strain 9R 5.5×10^5 failed to produce any growth; when 5.5×10^6 bacilli were plated L growth was formed but the yield was comparatively lower than that produced by a similar inoculum size of 9S.

In the induction of L colonies by the pour plate method strain 9R in the absence of serum produced only heteromorphic L colonies which appeared much earlier on the surface of the plates than those of strain 9S. The heteromorphic L colonies of strain 9R, however, contained more transitional and bacillary elements than those of 9S. Furthermore strain 9S produced L colonies irrespective of the presence of serum in the medium, whereas strain 9R produced L colonies only when serum was present. Strain 9S L colonies contained far fewer transitional forms than did strain 9R L colonies.

Another interstrain difference observed was the manner in which both strains 9S and 9R reacted to L transformation when serum was added to the M induction

medium. Although in both these strains addition of serum to the M medium depressed L transformation it was only in strain 9R that there was complete inhibition of L transformation.

In all these experiments penicillin was used as the inducing agent and the results would suggest that strain 9R possesses an inferior capacity to undergo L transformation under the influence of the agent. This may not always be the case, however, as can be seen in the experimentsin which both strains 9S and 9R cultivated anaerobically on a medium containing 200 units/ml of penicillin and 20 per cent. inactivated horse serum were able to produce classical as well as atypical L colonies. There were however,instances when strain 9S was inferior to 9R in undergoing L transformation. This was shown when the pH of the induction medium was altered. In the studies made on the effect of the hydrogen ion concentration on the L induction it was noted that at pH 6.7 strain 9R produced L growth containing no bacillary forms while strain 9S produced heteromorphic L growth with a number of untransformed bacillary elements. At pH 7.2 strain 9S produced L growth whereas strain 9R produced a slightly inferior growth and at pH 8.1 both the strains produced heteromorphic L growth, that produced by 9S containing marginally less transitional and bacillary forms. A similar finding has been reported

for E. coli strains by Seeberg and Brorson (1974) who observed that many strains of E. coli had different L transforming capabilities at different pH values.

With glycine as the inducing agent both 9S and 9R underwent L transformation readily when the medium contained 10 per cent. serum and 3 per cent. glycine but when the glycine concentration in the medium was halved the 9R strains produced a better quality of heteromorphic L growth than the 9S strains; moreover the heteromorphic L growth produced by strain 9S showed a tendency to revert earlier. It thus seemed that in the case of strain 9R glycine was slightly more effective in bringing about L transformation than in strain 9S particularly when serum was present in the medium.

Immune serum by itself did not bring about L transformation in either of the laboratory strains; however, it did induce a considerable degree of pleomorphism in 9S cultures but not in 9R cultures. This is probably due to the fact that the antiserum prepared against somatic O antigens was perhaps without effect on strain 9R which is mutationally O deficient.

The natural occurrence of L elements in the growth produced by field isolates on hypertonic medium containing no known L inciting agents made it difficult to compare the abilities of these isolates

to undergo L transformation with different L induction agents. In all eight field isolates examined between 5 and 30 per cent. of the cells in these cultures appeared as L elements depending on whether serum was present or not. Cultures of 9S and 9R never produced L elements in the absence of an L inciting agent. The spontaneous occurrence of transformed elements in bacillary cultures is well known. These forms occur naturally in Streptobacillus moniliformis, Fusiformis necrophorus, Haemophilus para-influenzae, Flavobacterium spp., Neisseria gonorrhoea, Vibrio cholerae, the viridans group of Streptococci, Bacillus subtilis and Bacteroides ruminicola (Klieneberger, 1935; Dienes, 1939a & b; 1940a; 1941; 1942 and 1946a & b; Carrere and Roux, 1953a; Hijmans and Dienes, 1955; Pachas and Dienes, 1968; Madoff, Burke and Dienes, 1967; Cheng, 1973). In the Salmonella spp. such a phenomenon has not been reported. However, Kagan et al. (1946a) have reported that L forms which had reverted to the bacillary state had occasionally retained their capacity to produce L forms spontaneously but this has not been verified in salmonellae, although it has been confirmed in the haemolytic group of streptococci. The fact that all eight field isolates had a natural inclination to produce L forms in hypertonic medium without the aid of an inducing agent and the absence of such capabilities in the laboratory strains has probably

important implications in the epidemiology of fowl typhoid. The author's view is that the field strains, being more recently isolated from clinical materials, had been conditioned during their in vivo existence to transform to their L phases during some stage of their persistence in their natural hosts and therefore possessed a natural capacity to revert to their L phases in the absence of an inducing agent. The inability of the laboratory strains (9S and 9R) to produce L elements spontaneously is probably due to the fact that these, being standard laboratory cultures, had undergone several passages on ordinary isotonic media and this, by a process of natural selection, would have eliminated those forms less suited to propagate under such environmental conditions.

The cultural conditions that favoured L transformation in the field strains were not studied to the same extent as in the laboratory strains but it was apparent that many of the environmental factors that favoured L transformation in strains 9S and 9R had a similar effect on the field strains; thus incubation of cultures at 30°C and the addition of serum to the medium also enhanced L transformation in the field isolates.

The effect of hydrogen ion concentration on L transformation was investigated on only two field strains ('846/71 and 154/71). In both of them at all three pH values investigated (6.7, 7.2 and 8.1) there was

production of L growth when 100 units/ml penicillin was incorporated in the medium. This is in contrast to the narrow pH requirements of 9S and 9R and possibly reflects the ease with which L forms can be induced in the field strains.

High concentrations of penicillin in the induction medium were not required to bring about complete L transformation in the field isolates. Of the two field strains tested (134/71 and 892/71) both produced L growth readily with as little as 50 units/ml penicillin provided serum was present in the induction medium. In the absence of serum even at concentrations of 100 units/ml penicillin strain 892/71 showed untransformed bacillary elements and transitional forms, showing that serum was an important requirement to achieve complete L transformation as has already been noted with the laboratory strains. At higher concentrations of penicillin (200 units/ml) but with 20 per cent. serum in the induction medium no differences were noted between the laboratory strains and the three field strains (37/74, 784/71 and 846/71 tested. The readiness with which some of the field strains transformed into their L phases at low penicillin concentrations suggests that in the field isolates the cell wall was possibly altered or slightly damaged so that it required an incitant of low intensity to cause further structural damage.

Glycine used at a concentration of 3 per cent. in a medium containing serum induced L transformation in all the 6 field strains tested (37/74, 154/71, 846/71, 131/71, 784/71 and 892/71) but in strains 37/74 and 784/71 L growth was noticeably retarded even when serum was present in the medium. The L growths obtained from the four remaining field isolates were comparable with those produced by strains 9S and 9R in AJA medium containing serum. These findings suggest that glycine at high concentrations (3 per cent.) may exert a toxic or inhibitory action on the L phases of some strains of Salm. gallinarum. When the concentration of glycine was reduced to 1.5 per cent. the field isolates produced L growth in contrast to the heteromorphic growth obtained from strains 9S and 9R. L growth or a high degree of L transformation was noticed as early as the 4th day of incubation in strains 846/71, 784/71 and 892/71 or as late as the 12th day of incubation in strain 154/71 or even longer in 37/74; in strain 131/71 L growth was established by the 14th day of incubation even in the absence of serum. The capacity of the field isolates to transform into their L phases in vast numbers at low glycine concentrations was considered significant.

The effects of immune serum on L transformation in the field strains were variable. L colonies or complete transformation of the bacillary elements into

their L phases were never produced with immune serum by itself in the laboratory strains or in the 5 field isolates tested. The effect of immune serum could only be assessed by comparing the microscopic composition of the growth produced on the test medium with that obtained on corresponding control medium. The interpretation of the results was particularly difficult in the field strains due to their capacity to produce L elements spontaneously. From the results of experiments conducted with immune serum it will be seen that in a number of field strains (846/71, 892/71, 595/72 and 1236/72) the effect of immune serum in bringing about increased L conversion was not significant. On the other hand in strain 784/71 the inclusion of rabbit serum (both immune and non-immune) appeared to suppress the appearance of naturally occurring L elements. Tulasne (1951) noted variations in L growth obtained in media containing bovine, rabbit, human and horse serum, Minck and Lavillaureix (1956) also stressed the importance of the source of serum indicating that human or horse serum gave the best results. In the Salmonella spp. rabbit serum had been reported to inhibit the development of L forms (Weinberger et al. 1950). The failure to increase L transformation in the bacillary cells of strains 846/71 and 595/72 despite the presence of antibodies and the inhibitory effects of immune and non-immune sera noticed in strain

784/71 could therefore be attributed merely to the presence of rabbit serum, particularly since serum by itself has been shown to be an important requirement in the development of L elements.

It has been shown that complement and immune sera acting on bacilli stimulate L transformation in the standard laboratory strain 9S, but in the case of two field isolates strain 892/71 produced the same effect as in 9S whereas in strain 1236/72, which had undergone S ————>R variation, L transformation was not stimulated. Although no definite conclusion can be drawn from the results with the two smooth strains and the single rough strain of Salm. gallinarum it nevertheless shows the different manner in which rough and smooth strains react to an immune humoral system. In earlier observations on strains 9S and 9R in a medium containing antiserum it was noted that 9S cultures developed a considerable degree of pleomorphism whereas the 9R cells in cultures did not appear to have reacted to the same degree. Since transformation from the bacillary state to the L phase is related to pleomorphism (Dienes 1942, 1946a & b) it could be construed that the pleomorphism observed in 9S cultures in the presence of immune serum was the first step tp L transformation and this appears to be so because, when bacilli, antiserum and complement were present together, L transformation occurred. On the other hand, the

inability of immune serum to bring about the same degree of pleomorphism in the rough strain (9R) indicated that the cells in these cultures were indifferent to the presence of antibodies and ; this is further exemplified in strain 1236/72 where the cells remained refractory to L conversion even in the presence of complement. The ineffectiveness of antibodies alone or in conjunction with complement to induce L transformation in rough strains of Salmonella gallinarum may be due to the lack of sufficiant amounts of the somatic O antigens in the rough variants.

The propagation of L colonies of strains 9S and 9R obtained with the aid of penicillin met with limited success and was not only dependent on the cultural environment but was also determined by the type of growth subcultured. These colonies rapidly reverted in the absence of penicillin and during prolonged incubation, indicating that the penicillin induced L forms of Salm. gallinarum of strains 9S and 9R were of the unstable type. Factors that favoured their induction were also important in their propagation; inclusion of serum and incubation of cultures at 30°C aided the maintenance of these L forms in subcultures. L colonies subcultured on AJA medium containing serum and penicillin in concentrations varying from 100 to 300 units/ml produced, in addition to L colonies, considerable numbers of transitional or bacillary elements. The

appearance of heteromorphic L colonies and revertant colonies could be attributed to (a) the use of aged L colonies in which reversion had already set in (b) to large numbers of incompletely transformed elements such as transitional filamentous or rod forms within the L colonies or (c) the progressive deterioration of penicillin in the propagating medium due to the presence of serum. Very often L colonies grew into the agar and these were only detected when the surface bacillary growth was removed; L colonies which were developing in the propagation medium were overrun by partially transformed elements (transitional forms) which had reverted to the bacillary state and these being able to multiply at a faster rate, overran the L colonies thus masking their presence. The use of very high concentration of penicillin to combat such reversions in the propagating medium often resulted in complete cessation of L growth denoting that penicillin at such high concentrations is toxic.

From the serial propagation experiments outlined in Tables 2-16 it is apparent that to maintain L growth in subculture the penicillin concentration had to be increased with each subculture in most instances even under ideal cultural conditions. There was thus a marked tendency for acquired resistance to penicillin to develop with each exposure to this agent. This resistance became more obvious when L or heteromorphic

L growth, exposed to high concentrations of penicillin (1,000 units/ml) and passaged once on medium containing 200 units/ml penicillin, produced large numbers of bacilli which remained refractory to L transformation when re-exposed to high penicillin concentrations (1,000 units/ml).

The failure to maintain L forms of Salm. gallinarum in subcultures in the presence of a constant amount of penicillin which was sufficient initially to induce L transformation is contrary to the observations reported in other bacterial species. Bertolani et al. (1975) were able to maintain L forms of Pseudomonas aeruginosa in subcultures in a liquid medium containing 8,000 µg/ml carbenicillin without causing reversion. Ward and Martin (1962), working with 96 bacterial strains of different species, were able to obtain L growth in 16 strains with penicillin and cycloserine and to maintain them in serial transfers on media containing either of the antibiotics. Silberstein (1953) noted that it was possible to maintain unstable L forms of Proteus spp. for 8 months in the presence of penicillin provided subcultures were made at appropriate intervals. He also reported that L growth subcultured on medium containing higher or lower concentrations of penicillin than that used forthe initial isolation produced no untoward effects. The increasing requirement of penicillin in maintaining L growth in Salm. gallinarum could not

be due solely to the decreasing activity of penicillin in the propagating medium during prolonged incubation in the presence of serum proteins. A more possible explanation is that the initial exposure to penicillin caused L transformation in all cells but their subsequent ability to remain in the L phase in the presence of penicillin could have varied with the individual cells in the population. The present work suggests therefore that while all the cells transformed initially there were many in which transformation was of a transitory nature in that they were able to revert in the presence of the original level of penicillin that induced them. Such a mechanism of reversion could also be responsible for the emergence of 'persistors' (bacillary forms which resisted L transformation on subsequent exposure to penicillin). Furthermore, it was observed that when L and heteromorphic L colonies were made to revert completely and then subcultured on to media containing penicillin they resisted transformation. The present studies do not therefore corroborate the findings of other workers that revertant forms possessed greater capacity to form L colonies (Kagan et al. 1962, Kagan et al., 1964a; Stewart and Wright, 1969; Dienes, 1970b; Madoff, 1970; Pachas and Currid, 1974).

Kagan et al. (1963) advocated the use of a method by which they were able to transform heteromorphic

growth into L colonies by successive passages in medium containing higher concentrations of penicillin. Using their method, it was possible to convert heteromorphic L colonies and revertant L colonies (i.e. L colonies in which reversion had set in but was not complete) of Salm. gallinarum into L colonies by serial transfers on medium containing increasing amounts of penicillin.

Continued passage of L and heteromorphic L colonies on media containing 1,000 or more units/ml penicillin and 20 per cent. serum produced, in addition to L elements, abnormal forms (T elements) consisting of large fusiform and bulbous elements, aster-like bodies and swollen braching filamentous forms (see plate 30). These were difficult to differentiate from transitional elements except that they appeared only in AJA or NAJA medium containing very high concentrations of penicillin and 20 per cent. serum and occasionally in medium containing 10 per cent. serum or sometimes even in medium containing 400 units/ml penicillin and 20 per cent. serum. These elements were also encountered when L growth of Salm. gallinarum strain 9S was adapted to grow in liquid medium. It is possible that the 'T' elements seen in Salm. gallinarum L cultures are akin to the altered L forms observed by Dienes (1970b) in L cultures of Salmonella and Proteus spp. that had been continuously passaged on medium containing penicillin. Not only did they

resemble the altered L forms of Dienes morphologically but they also possessed many common cultural characteristics and emerged as a result of prolonged passage of L growth on media containing penicillin. Dienes suggested that these altered L cultures were a fixed transition between the B type L forms and bacteria. In the absence of any other report on the presence of such elements no definite comments can be made of the 'T' elements in Salm. gallinarum, except to consider them as another variety of elements found in L cultures.

Throughout the serial transfers of L and heteromorphic L colonies of Salm. gallinarum it was the rough (9R) rather than the smooth strain (9S) that showed a greater tendency to revert under unfavourable conditions. This was anticipated as strain 9R was generally less amenable to L conversion than strain 9S particularly when penicillin was used as an inducing agent.

During the course of the present studies it was noticed that L and other heteromorphic L growth contaminated by yeasts and moulds showed more reversionary changes than those in uncontaminated plates. It must be pointed out however, that contamination of the plates generally occurred during the periods of prolonged incubation and therefore reversionary changes were already taking place in these cultures but that they became accelerated only in plates containing the contaminant growth. Dienes (1970a)

has reported the appearance of bacterial forms in L cultures of Haemophilus influenzae under the influence of Bacillus Y and,likewise, Madoff (1970) found that L colonies in the proximity of a fungal growth shows reversion. In the L and heteromorphic L colonies of Salm. gallinarum it is not possible to say that the contaminant yeast colonies initiated reversion but they did appear to accelerate the process of reversion.

Glycine induced heteromorphic L growth of strain 9R could be propagated without reversion far more successfully than that induced with penicillin, although reversion did occur when glycine was removed, the process being much slower. The heteromorphic L growth could be maintained however, without reversion if the glycine and serum content were maintained at the levels used to induce them. This was contrary to that observed with the penicillin-induced heteromorphic L growth which required higher concentrations of penicillin to prevent it from reverting with each subculture.

The ability of the glycine-induced heteromorphic L growth to maintain its colony status on subculture suggested that L forms, transitional elements (partially transformed bodies) and bacillary forms found in these heteromorphic L colonies had not acquired resistance and also denoted that low levels of glycine (i.e.

1.5 per cent.) were not sufficiently potent in the strain 9R to convert the bacilli and partially transform cells fully into L elements. Increasing the glycine concentration in the propagation media had the effect of transforming heteromorphic growth into L growth. Using a smooth strain (field isolate 846/71) the results were far more convincing. L colonies produced by this strain on a medium containing 10 per cent. serum and 1.5 per cent. glycine could be transferred to a propagating medium of similar composition or to one containing higher amounts of glycine (3 per cent.) without reversion being caused. In Salm. typhi, L forms have been also induced with glycine but these reverted on media containing no glycine (Diena et al. 1965). These workers attempted to combat reversion by subculturing their organisms on a solid medium containing glycine, but revertants still appeared. They condluded that glycine-induced L forms were of the unstable variety and ascribed the emergence of revertants to an acquired resistance of glycine. The present studies with Salm. gallinarum confirms these findings only in so far as instability of the L forms is concerned, but there is little evidence to suggest that the bacillary or L elements of Salm. gallinarum acquired resistance after their initial exposure to glycine.

The gross morphology of L colonies has often been

used as an important criterion for their identification. In Salm. gallinarum the growth of L elements on solid medium was manifested by a variety of colonial forms (see plates 2, 3, 6, 14, 19, 20, 23, 34, 39 and 51).

The development of classical L colonies of Salm. gallinarum with a dark central core surrounded by a lighter periphery (see plates 2, 3, 20 and 39)was largely determined by the physical characteristics of the medium as has been reported by Razin and Oliver (1961). Between pH values of 7.2 and 8.0 both classical L colonies or a confluent growth were produced depending upon the softness of the agar and the presence of free moisture. At pH 8.1 there was a marked tendency to form colonies with a classical morphology whereas at pH 6.7 confluent L growth was produced.

The surface growth of L colonies of Salm. gallinarum (see plates 14 & 51) showed very little or no penetration of the agar and often was confluent when growth was heavy or occurred in patches when it was poor or during initial stages of development. The surface type of L growth was by far the most common type obtained during the present studies. This was due to the abundance of free surface moisture and the softness of the solid medium used in the present series of experiments. In E.coli the tonicity of the medium, the pH and the nature and concentration of the antibiotic used influenced the appearance of the colonies (Seeberg

and Brorson, 1974) but in Salm. gallinarum neither the nature of the inducing agent nor its concentration was a determining factor in the formation of L colonies with either a classical or confluent morphology.

In addition to the confluent/patchy surface L growth and L colonies with a classicalmorphology, a number of atypical types of L growth of Salm. gallinarum were encountered (see plates 6, 19, 23 and 34). The large globular L colonies (plate 19) were produced in media containing more than 400 units/ml of penicillin and 20 per cent. serum. These colonies were probably the result of L elements growing within the depths of the agar aided by the softness of the medium. The micro L colonies (plates 6 and 7) and the 'bow-tie' velvety L colonies (plate 34) were only occasionally encountered and no satisfactory explanation can be given as to what factors influenced their unusual gross appearance. The 'bow-tie' L colonies were only produced by strain 9R on medium containing 3 per cent. glycine with or without serum. The occurrence of atypical L colonies is not unusual; it has been reported to occur in Clostridium perfringens and Erysipelothrix rhusiopathiae (Kawatomari, 1958; Pachas and Currid, 1974) but the appearance of such colonies has not been reported in Salmonella spp. except by Mattman et al. (1969) in Salm. pullorum.

It was of interest to note that very often colonies

with a classical L colony morphology were not L colonies but heteromorphic L colonies or colonies in the process of reversion (see plates 8, 9, 18, 28 and 29). Likewise the confluent L and heteromorphic L growth could easily be mistaken for bacillary growth on gross appearance. The present studies therefore show that the identification of L growth cannot be based strictly on gross colony morphology.

The composition of the L phase growth of Salm. gallinarum seen under the highest magnifications of the light microscope consists of polymorphous bodies which were not fundamentally different from the basic elements present in L colonies derived from other species of bacteria described by various other workers. Hijmans et al. (1969) have broadly separated L elements into 3 groups, namely large bodies, granules and elementary corpuscles. According to them large bodies consist of rounded, swollen forms whose sizes range from 1 to 50 μm. On the other hand, workers have just defined L forms as round bodies arising from bacilli under the influence of L inducing agents (Roberts et al., 1974).

In the L phase growth of Salm. gallinarum it has been found possible to further classify the round bodies into 5 subgroups viz. large, round, pale-staining bodies, large, medium-sized, small and tiny spherical elements. The large, round, pale-staining bodies constitute those forms which have a diameter of 25μm

or more and stain faintly with Dienes' stain. They are further characterised by their extreme susceptibility to mechanical distortion and their total inability to withstand Gram's staining procedures. Their early appearance in large numbers in L and heteromorphic L growth, their frequent association with untransformed bacilli and transitional filamentous forms and their rapid disappearance from L colonies probably indicates that they arise directly from bacillary and transitional forms. This conclusion is in agreement with the findings of Dienes (1946a & b) who suggested that large bodies develop from bacilli and filaments.

Bacilli were often noticed within the large round pale-staining bodies and there is also evidence in the present studies to show the emergence of bundles of bacilli from these giant forms in the process of complete disintegration (see plate 40). Likewise granules were also present in these bodies and the appearance of tiny spherical elements in the degenerating large round pale-staining bodies is a convincing proof that these bodies possess the dual potential of either reproducing bacteria or smaller L elements (Dienes, 1946a &b, 1968; Dienes and Bullivant, 1967). There is no evidence, however, to suggest that these large bodies reproduce as large bodies as reported by Bandur and Dienes (1963); nor did they reproduce as filaments as described by Minck and Lavillaureix (1956) and Dienes (1970c).

It was not possible to ascertain the origin of the large and medium-sized spherical elements. These bodies, measuring from 5-20μm, were also present during the early stages of L or heteromorphic L colony development and were often found intermingled with filamentous bodies (see plate 37) sometimes being agranular but more frequently containing granules. They resembled the small and tiny spherical elements in their ability at times to withstand the Gram's staining during certain stages of their development, their moderate resistance to mechanical distortion and their occasional agranular appearance. However, the large and medium-sized spherical elements disappear earlier in reverting cultures than the smaller L elements. These findings suggest that they may be intermediate stages between the smaller L elements and the large round pale-staining bodies. It is also probable that they may have emerged directly from bacilli or been produced by the detachment of the herniations occurring on the filaments.

The small and tiny spherical elements measuring less than 5μm are often the last bodies to appear in L cultures and the last L elements to disappear in reverting cultures. Like the granules these smaller L elements are intensely stained by Dienes' staining method. They appear within disintegrating large round pale-staining bodies and therefore it is possible that these small elements could be the result of the growth

of the granules or the fusion of the intracellular coarse granules found in large round pale-staining bodies; but proof of this is not entirely lacking in the present studies as can be seen from the analysis of the findings of the filtration studies reported below. The presence of unusually large numbers of small and tiny spherical elements in bacillary cultures of the field strains on hypertonic media devoid of penicillin is baffling. No satisfactory explanation can be offered except to speculate that these isolates had been exposed, during their in vivo existence, to L transforming agents which would have produced a large number of bacillary forms having undergone some measurable degree of cell wall damage.

The distortion of morphology and deformation of cells brought about by routine bacteriological procedures has been pointed out by Klieneberger-Nobel (1960) but the present studies indicate that the various elements present in L cultures differ in their ability to resist mechanical deformation; likewise, the ability of some of the elements to withstand Gram's staining procedures (which entail the use of hypotonic solutions) demonstrates their resistance to osmotic lysis. Furthermore, it has been shown that L and heteromorphic L growth which has been serially propagated on a solid medium can be suspended in normal saline without loss of viability, whereas in newly produced L growth a

large proportion of the cells are sensitive to osmotic lysis and mechanical deformation. Razin and Argaman (1963) have reported that L forms and spheroplasts are more resistant to osmotic lysis than protoplasts. They attributed this to the presence of a modified cell wall in L forms and spheroplasts. It is possible therefore that the ability of some of the L forms of Salm. gallinarum to withstand osmotic and mechanical damage can be related to the presence of a modified cell wall inherently possessed or acquired during their later stages of development.

In vitro induction of spheroplasts of Salmonella spp. in a liquid medium has been reported by Jeynes (1957), Diena et al. (1964) and Federova (1965). All these workers used glycine or versene-lysozyme to induce spheroplasts. Salm. gallinarum spheroplasts were easily produced by adding penicillin to growing bacillary forms in a liquid medium of high osmolarity. Spheroplast formation was noticed between 18-24 hours following the addition of penicillin, with numbers increasing at the 48th hour and reaching a peak between the 6th to 10th days of incubation. No spheroplast formation was noticed within the first 5 hours of adding penicillin. These results differ from those reported by the above workers. Federova (1965) reported that in Salm. typhimurium complete transformation of bacilli into spheroplasts occurred within 20-30 minutes following exposure to versene-lysozyme and with glycine a 100 per cent. conversion

into spheroplasts was obtained after 18 hours' incubation. Although the results obtained with Salm. gallinarum cannot be compared with those obtained in Salm. typhimurium it is possible however that either penicillin was inferior to versene-lysozyme and glycine in bringing about cell wall damage or, more likely, that the bacillary forms of Salm. gallinarum were slow to react to the presence of penicillin and that a more prolonged contact was essential to bring about a degree of cell wall damage sufficiently severe to cause them to convert.

The differences noticed between spheroplasts produced directly from bacillary cells and those obtained from heteromorphic and reverting L cultures in their ability to persist in the liquid medium possibly indicate basic differences in the cell wall structure of these types of spheroplasts. Another point of interest is the appearance of yeast-like bodies, racquet and dumb-bell forms in aged PW cultures of Salm. gallinarum in which no penicillin was present and the presence of occasional spheroplasts in similar AJB cultures. The presence of these odd forms in PW cultures might be an attempt on the part of bacillary forms to react to the presence of toxic metabolites produced in the medium but because of the isotonicity of the PW medium they were unable to form spheroplasts, whereas in AJB under similar circumstances spheroplasts were occasionally produced

because of the hypertonicity.

That the spheroplasts of Salm. gallinarum were osmotically sensitive was apparent from the fact that such bodies could not be obtained from PW cultures to which penicillin was added; that these spheroplasts were unstable cell wall defective variants is indicated by the fact that bacilli reappeared in large numbers with prolonged incubation.

It could not be established with certainty whether spheroplasts were capable of serial replication but it was probable they were so because the number of spheroplasts produced initialy from broth cultures of bacilli was dependent on the amount of penicillin used, but the maximum spheroplast formation (which occurred between 5-10th day of incubation) was achieved irrespective of the concentration of penicillin in the medium. Furthermore, one would have anticipated that during the period of high spheroplast formation there would have been a progressive loss of penicillin activity due to prolonged incubation at 37^{o} and consequently a considerable loss of spheroplast forming power. Therefore it was unlikely that dormant bacilli were being converted into spheroplasts in such large numbers.

In the method outlined for the production of stable L growth of Salm. gallinarum strains 9S and 9R (see pages 359 - 364) it is important that only L colonies

produced and passaged at least once on a solid medium containing high concentration of penicillin (1,000 units/ml) could be adapted to grow in a liquid medium. This preliminary exposure of the L colonies to such high concentrations of penicillin on a solid medium was found to be necessary to enable the L elements to grow in the liquid medium containing similar or higher concentrations of penicillin. The penicillin concentration in the liquid medium was maintained at a high level during the initial stages of stabilisation to forstall any reversion. Between 4-6 serial passages in a liquid medium containing penicillin were necessary to ensure stabilisation. The higher penicillin requirement in the liquid medium for strain 9R was not unexpected as it had all along shown considerable resistance to L transformation.

The gross appearance of the stabilised L growth of Salm. gallinarum in the liquid medium resembled the L variant growth of Streptobacillus moniliformis and Erysipelothrix rhusiopathiae in fluid cultures (Klieneberger, 1936; Klieneberger-Nobel, 1960, 1962; Heilman, 1941a; Pachas and Currid, 1974), although Dienes (1970b) had reported that the altered L variants of Salmonella and Proteus produced mucoid material in liquid media, this was not evident in the liquid L cultures of Salm. gallinarum.

The microscopical appearance of the stabilised L

growth showed a variety of elements many of which had a striking resemblance to the altered L forms of Salmonella and Proteus (Dienes, 1970b). The presence of such varied morphological types in these cultures in addition to the spherical bodies and granules raises doubts as to whether they could be the type C of L forms described by Dienes (1970b).

Although serum was found to be necessary for the induction and propagation of L forms on solid media as well as during the initial stages of stabilisation, it was not a critical requirement for the propagation of the stabilised L forms in liquid medium. The elimination of serum requirement by the stabilised L forms of Salm. gallinarum is an important finding in that it can without hindrance, permit the immunological characterisation of these cell wall defective variants. On the other hand, the tendency of some of these elements to revert at 37°C (although they could be induced at this temperature) is a definite disadvantage in the evaluation of the pathogenic potentials of these organisms in animal hosts.

This method of producing stable L growth in Salm. gallinarum in the present studies does not appear to have been described previously and perhaps merits consideration for use in those organisms which resist stabilisation on a solid medium. The present findings are also unique in that an organism that did not produce

a stable growth on a solid medium could be stabilised in a liquid medium. A possible explanation is that removal of the cell wall deprives the L forms of a primer of a feedback reaction chain that is required for septation and cell wall formation (Landman and Halle, 1963). In stable L growth cell wall disintegration proceeds under the influence of an L transforming agent to a point where the feedback system is permanently damaged; reinitiation of the feedback system whereby the cell walls are resynthesised (i.e. reversion to the bacillary state) is only possible if facilitated by the presence of hard agar which physically simulates the primer (Landman, 1968). In Salm. gallinarum, however, it is postulated that the mere presence of a gelling agent (irrespective of the agar concentration) is sufficient to provide the necessary environement for cell wall accretion, thus explaining the inability of these L forms to produce a stable growth on solid media.

The present studies also showed that the stabilised growth of Salm. gallinarum can be serially propagated in liquid medium contrary to the reports that agar is crucial for L form multiplication (Lederberg & St.Clair, 1958; Altenbern & Landman, 1960). However it should be pointed out that L forms of Salm. gallinarum, like the mutant forms of Proteus shared the ability to produce surface L colonies without penetration of the agar

(Altenbern & Landman, 1960) indicating that such penetration into agar was not necessary for growth and multiplication and since these two workers have shown that the centreless surface mutant L colonies of Proteus were capable of multiplication in a liquid medium it is probable that the L forms of Salm. gallinarum could have similar reproductive capabilities.

The filterability of the L phases of bacteria has been demonstrated by a number of workers (Klieneberger, 1936; Silberstein, 1953; Carrere et al., 1954; Kellenberger et al., 1956; Tulasne and Lavillaureix, 1958; Rada, 1959; Panos et al., 1960; Williams, 1963; Mortimer, 1965; Molander et al., 1965; Coussons & Cole, 1968; van Boven et al., 1968 and Roberts, 1968). The positive filtration results obtained with Salm. gallinarum confirm the findings of these workers as far as the filterability of L phases is concerned. On the other hand the present results also indicate that L cultures are only filterable at certain stages of their development and not all elements present in L cultures are filterable. This was well demonstrated when suspensions of young heteromorphic L growth of strains 9S and 9R in a hypertonic medium after being filtered through a 0.45µm A.P.D. Millipore filter membrane failed to produce any growth on a solid medium, whereas an aged reverting L colony of strain 9R, consisting of bacillary forms and L elements (chiefly

the tiny spherical elements) filtered under identical conditions, produced L colonies on a solid medium. As bacilli has been shown not to pass through a filter membrane of similar A.P.D. in the present experiments it is logical to conclude that only L elements could have passed through the filter membrane. It is unlikely that transitional forms could have made their way through filters of such small pore dimensions which retain bacteria; the transitional elements were not only larger and more distended than bacteria but also were very often characterised by the presence of saccular or spherical distention to enormous sizes and this would have precluded their entry via these filter membranes. Furthermore if transitional elements were able to pass through these filters, a positive filtration result would have been anticipated with the filtrates from the young heteromorphic L colonies which generally contain large numbers of transitional elements. The differences in the filtration results obtained from the young heteromorphic L colonies and the aged reverting L colonies might possibly be attributed to the microscopic composition of these two colony types. It has already been pointed out that the smaller spherical bodies generally appeared in large numbers in the later stages of L or heteromorphic L colony development and were often the last cellular L elements to disappear from reverting colonies. Since

tiny spherical elements were present in the aged reverting L growth used for the filtration experiments it would seem that these elements are able to pass through the filters and initiate growth on the solid medium. On the basis of these findings it is possible that the inconsistent results obtained by Heilman (1941a) can be ascribed to the developmental stage when the L cultures were subjected to filtration.

Lederberg and St. Clair (1958) and Weibull and Lundin (1962) pointed out that the plasticity of the L elements should be taken into account in the interpretation of filtration experiments, but in the present work carried out with L phase variants of Salm. gallinarum it is unlikely that the larger L elements were able to pass through a filter pore size of 0.45μm A.P.D. for, if they were able to do so, a positive filtration result would have been obtained from the filtrates of the heteromorphic L colonies. On the other hand it could be argued that the plating medium containing large amounts of penicillin (1,000 units/ml) was exceedingly toxic for the larger L elements. This, however, is highly improbable since, in the same experiments, it was shown that unfiltered suspensions of the heteromorphic L growth of 9S plated out on a similar medium produced a patchy granular L growth which contained many well developed and granulated L elements of the larger varieties.

In the indirect method of assessing the filterability (i.e. placing bacillary or L colonies face downwards on filter membranes resting on media and demonstrating growth beneath the filters) it was demonstrated that L forms of Salm. gallinarum were able to grow through filter pores 0.6 and 0.8μm A.P.D. and form colonies on a hypertonic medium containing penicillin. The differences in the microscopic composition of growth produced beneath the 0.6 and 0.8μm A.P.D. filters was of interest. In the growth produced under the 0.6μm filter there were L elements (preponderance of tiny spherical elements) than bacillary elements whereas in the growth produced beneath the 0.8μm filter the L:bacilli ratio was reversed. The fact that bacillary cultures of Salm. gallinarum under similar conditions are unable to grow through these filters and form colonies and that the L colonies contained no bacilli suggests the possibility that these bacillary forms arose from the L elements which had traversed the filters and, after a time, had reverted. The basis for arriving at this conclusion is as follows:-

a. L colonies used for this experiment were obtained from a hypertonic medium containing high amounts of penicillin (1,000 units/ml) but these colonies rested on filter membranes that were placed on a similar medium containing 200 units/ml penicillin thus favouring reversion of some of the L elements which might have

passed through the filter pores.

b. Some of the larger L elements could have passed through the filters of 0.6 and 0.8μm A.P.D. because of their plasticity, although they were unable to do so through a 0.45μm A.P.D. filter membrane.

c. The L elements that had succeeded in traversing these filters would probably have grown and enlarged into larger spherical elements and these in turn could have further distended or grown into large round pale-staining bodies.

d. The large round pale-staining bodies have been shown to produce bacilli.

The preponderance of L elements in the growth produced under the 0.6μm filter and the reduced numbers in the growth beneath the 0.8μm filter can be explained on the basis that the 0.8μm filters allowed the passage of relatively more of the larger L elements and therefore these matured and reverted earlier than the smaller L elements which, having passed through the 0.6μm filters, took more time to grow through the developmental stages to produce the bacillary forms. Furthermore, many of the larger L elements that had passed through the 0.8μm filters would have been retained by the 0.6μm filters.

L forms of Salm. gallinarum grown in a liquid medium were unable to pass through filter pore sizes of 0.22 and 0.45μm by the direct filtration techniques.

The tiny spherical elements present in the liquid L cultures were not filterable through filter membranes of 0.45μm A.P.D. whereas those originating from a solid medium were shown to pass through filter membranes of similar pore dimensions. This discrepancy could possibly be related to the tendency of L forms in liquid media to produce flaky growth and therefore it is suggested that many of the tiny spherical elements were clumped together preventing them from passing through filters with small porosities.

There were small variations between the biochemical reactions of the 10 bacillary isolates of Salm. gallinarum but these were nothing more than normal inter-strain differences. The biochemical reactions of the L forms generally resembled those of their parent forms except for minor differences in the ability to break down a few sugars and the production of H_2S. These observations are in agreement with the findings of most other workers who investigated the physiological and biochemical properties of L forms (Heilman, 1941b; Weinberger et al., 1950; Minck, 1952; Kandler et al. 1956; Robert & Wittler, 1966; Cohen et al., 1968; Edman et al., 1968). The utilisation of carbohydrates was generally slower in the L forms than in the bacillary phases from which they were derived. An observation of a similar nature has been reported by Bertolani et al. (1975).

Kagan and Levashov (1957) have reported that

Salm.typhi revertant arising from their L phases could be separated on the basis of their biochemical characteristics into three groups viz. (a) revertants resembling the parent forms in their biochemical properties (b) revertants that decompose one or two sugars not attacked by the parental forms and (c) revertants that were biochemically inert. In Salm. gallinarum there are, however, no marked differences between the biochemical properties of the revertants and the original parental bacillary forms, nor were there any significant variations between revertants and the L phases from which they were derived.

Studies on the ultrastructure of the bacillary forms of Salm. gallinarum showed the cell wall of this organism to consist of a multilayered structure closely resembling the cell walls of other Gram negative bacteria described by a number of workers (Kellenberger & Ryter, 1958; Glauert, 1962; Bladen & Waters, 1963; Claus & Roth, 1964; de Petris 1965, 1967; Hofschneider & Martin, 1968; Murray, 1968).

The cell wall layers of the bacillary forms of Salm. gallinarum could be identified with that of E. coli described by de Petris (1965, 1967). According to the terminology of this author the cell wall of E. coli proceeding from outside to inside consists of an L membrane (electron dense-light-dense layer designated as l_1, l_2 and l_3), a G layer (outer g_1 which is electron

transparent and an inner g_2 component which is electron dense) and an M layer which is adjacent to the cytoplasmic membrane and electron transparent. In the bacillary forms of Salm. gallinarum, however, in addition to the 3 electron dense lines an extra electron dense line is present internally to the three dense lines (see plate 64), i.e. inside the g_2 layer of de Petris (1967). This fourth electron dense line is less intensely stained than the other lines and is not visible at magnifications of x80,000 or less. It is unlikely that this fourth dense line is an artefact as it had the appearance of forming a definite layer. It is also improbable that this line could represent the M layer which has been shown to be electron transparent (de Petris, 1967); nor can it be regarded as a detachment of the cytoplasmic membrane as this layer shows the characteristic "unit" membrane structure at the place where the fourth electron dense line is present. It is not possible therefore to identify this extra electron dense line with any of the known components or layers of the cell wall. It is felt that it should be considered as part of the cell wall because this line lies well outside the triple layered cytoplasmic membrane.

Ultrathin sections of L forms of Salm. gallinarum grown both in solid and liquid media clearly showed that these cell wall defective variants could be classified on the basis of their limiting membrane into three main

types viz. (a) those whose cell contents were bounded only by a cytoplasmic membrane, (b) L forms that possessed in addition to the cytoplasmic membrane a modified cell wall and in (c) cell wall defective variants in which small segments of the cell wall were found still attached to the cells but major portions of the cell contents were enclosed by a cytoplasmic membrane or a modified cell wall.

L forms of the first type appear to be similar in structure to the protoplast or Type A L forms described by Weibull (1965, 1968), Dienes and Bullivant (1968), Hofschneider and Martin (1968) and Delmonty, Robaye and Calberg-Bacq (1973). In the present work L forms of the second variety in which a modified cell wall is present probably correspond to the Type B unstable L form (Dienes & Bullivant, 1968; Delmonty et al., 1973), to the stable spheroplast type L form of Proteus described by Hofschneider and Martin (1968) and to the L forms of Brucella abortus (Hatten et al., 1969). The only other report in which segements or intermittent stretches of the cell wall remain attached to the L cells (corresponding to the third type of cell wall defective variant) is that of Hofschneider and Martin (1968), who found that some of their unstable spheroplast L forms of Proteus possessed an intact cytoplasmic membrane whereas the outer cell wall was extensively disorganised and only short stretches of the cell wall were preserved. The present studies on the L forms of Salm. gallinarum therefore

corroborate their findings.

Vesicular and small dense bodies whose sizes ranged from 70-450nm found in L cultures of Salm. gallinarum did not present any characteristic arrangement either within or outside the L elements and therefore it is difficult to assign any particular role to them. Undoubtedly some of the empty vesicular bodies are nothing but membranous debris. On the other hand, many of the vesicular and small dense bodies containing ribosomal particles cannot be dismissed as fragments of damaged membranes as these resemble some of the submicroscopic elements present in L cultures of Proteus, Salmonella and Brucella (Dienes & Bullivant, 1967, 1968; Dienes et al., 1968; Hatten et al., 1969). The small dense bodies packed with ribosomal particles are very similar morphologically to the elementary corpuscles described by some of the above workers and therefore it is possible that they may have a reproductive role in the genesis of some of the L elements.

The cell wall of the transitional filamentous forms resembles that of the bacillary forms except that at their extremities there was evidence of cell wall damage and loss of cell wall layers with a concomitant spherical distension of the filament where cell wall damage was apparent. It is therefore suggested that these forms be rightly designated as transitional forms and not regarded merely as filaments since they

represent a stage towards the transition of a rod-shaped organism to that of a cell wall defective variant form.